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The Genetic Basis of Fruit Characteristics and Fruit Quality Traits in Mandarin Hybrids

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DEDICATION

I dedicate this thesis to my mother, Nebiye Celikli who devoted her life to me, showed me how a woman is strong on her own, taught me not to give up, and always supported and encouraged me throughout my life.

Table of Contents

List of Figures

List of Tables

INTRODUCTION

Citrus is a genus, which is evergreen trees, that belongs to the Rutaceae family, and is grown in tropical and subtropical countries in a belt from 40 ^{0} North Latitude to 40 ^{0} South Latitude (Appelhans et al. 2021). Citrus is generally thought to have originated in South Asia's tropical and subtropical areas, including China, India, and the Malay Archipelago. After domestication, it was distributed from these regions to the rest of the world as well (Gmitter and Hu 1990; Federici et al. 1998; Nicolosi et al. 2000).

Before giving a scientific name to Citrus or before Linnaeus's classification, it was named "Kedros." The word "Citrus" emanates from "Kedros," the Latin form. Kedros is originally a Greek word for fragrant trees such as cedar, cypress, and pine. Citrus leaves and fruits had their specific fragrance, and the smell of Citrus leaves and fruits resembled cedar. For this reason, Citrus fruits were named "Kedros" (Spiegel-Roy and Goldschmidt 1996; Deng et al. 2020). Although the history and geographical origin of Citrus remain unclear, there are different opinions about that (Khan 2007; Talon, Caruso, and Gmitter 2020). The earliest known reference about the Citrus origin is the myth of Hesperides's Golden Apples in Greek mythology. According to the legend, golden-colored apples were hidden in Hesperides' garden. The son of Heracles, Greek Herakles, Roman Hercules, Zeus (father), and the mortal Alcmene (mother) had to labor to take three golden apples from the Hesperides' garden. A dragon guarded this garden. Hercules killed the dragon and took the apples. It was believed that if anyone ate these apples, they could give immortal life to the person. There was a rumor that Gaia gave

these apples or Citrus fruits at Hera and Zeus's wedding. Due to the Hesperides' golden apples legend, the Greek botanical name of Citruses is “πορτοκάλι," which is "Hesperidoeidē.” Therefore, there was doubt whether these golden apples represent today's apples or other fruits. In those times, the word "apple" used to be given as a name to all fruits except for berries. Although there is a rumor that these apples were quinces, most opinions are that these mentioned golden apples may be Citrus fruits (Khan 2007; Talon, Caruso, and Gmitter 2020).

Even if its origin is not known exactly, Citrus fruits are one of the most grown fruits in the world today, with production exceeding 113 million tons (Mt) year $^{-1}$ from about 10 million ha in 2020 (FAOSTAT, 2020). Sweet oranges (*Citrus sinensis* (L.) Osb.), mandarins (*Citrus reticulata* Blanco), satsumas (*Citrus unshiu* (Mak.) Marc), Clementines (*Citrus clementina* Hort.), lemons (*Citrus lemon* (L.) Burn) and grapefruits (*Citrus* × *paradisi* or *Citrus paradisi* Macf.) are cultivars that have commercial importance in the world (Blasco, Cubero, and Moltó 2016); Ma et. al, 2020, FAOSTAT, 2020). China, Brazil, and the US are generally among the leading Citrus-producing countries (Crifò et al. 2012; Turner and Burri 2013; Ballistreri et al. 2019; Ma et al. 2020; Visvanathan and Williamson 2021), but this differs slightly when we look at the Citrus species separately in terms of their production quantity. According to FAOSTAT (2020) values, sweet oranges and sour oranges were categorized within the orange group by indicating the production amount of orange. The most produced Citrus fruits in the world, measured by quantity, were oranges with 75 million tons. Mandarins are the second most-produced among Citrus cultivars following orange production, with 38 million tons.

Tangerine, mandarin, Clementine, and Satsuma were evaluated in mandarin groups. Brazil, India, and China are the leader countries in total orange production. China, Spain, and Turkey are the places where the most mandarin is produced around the world. Mandarins are the most produced Citrus species after oranges. It is seen that the production amount of mandarin has increased since 2016. Even though the mandarin production area (3,055,867 ha) decreased in 2020 compared to the previous year (3,047,850 ha), there was an increase in mandarin production in 2020. For the last five years, China, Spain, and Turkey have maintained their leadership in mandarin production, respectively (FAOSTAT, 2020).

Thanks to the taste and aroma that Citrus fruits have, Citrus fruits are consumed as food such as fresh fruit, juice, and jam. Furthermore, Citrus varieties are used in food, cosmetics, perfumery, and chemoprophylactic drug production in the pharmaceutical industry. In addition, Citrus species are beneficial for human health due to their phytochemicals and nutrients. The peel and pulp of Citrus cultivars contain ascorbic acid (vitamin C), alkaloids, coumarins, limonoids, carotenoids, and flavonoids such as anthocyanins. These phytochemicals with biological activities are essential for human health, and they help to prevent human degenerative diseases, including cancer, aging, cardiovascular diseases, inflammation, and diabetes II (Crifò et. al, 2011; Turner & Burri, 2013; Ballistreri et. al, 2019; Ma et. al, 2020; Visvanathan & Williamson, 2021). Citrus fruits are among the most consumed fruits in the world due to their beneficial properties for health and their taste and aroma components. For this reason, it is important to

develop new varieties with higher fruit quality and meet consumer demands with Citrus breeding.

One of the most important goals of Citrus breeding is to produce and improve Citrus species with desired properties for global marketing. To obtain the selected properties, fruit quality characteristics must be improved. In Citrus fruits, especially mandarins, fruit size, taste, seedlessness, peelability, and color are critical fruit quality traits. These fruit characteristics are complex, and they can be affected by genetics (G), environment (E), genetics and environment interaction (Gx E). To develop new varieties with desired properties for mandarin breeding, the genetic basis of fruit quality characteristics should be well understood. Modern genetics and modern biology tools such as molecular markers, linkage mapping, and quantitative trait locus (QTL) analyses are performed for genetic studies in mandarins (Şahin-Çevik and Moore 2012; Omura and Shimada 2016; Yu, Chen, and Gmitter 2016; Goldenberg et al. 2018; Imai et al. 2018). Genetic map building with quantitative trait loci (QTL) analysis is needed to identify possible candidate genes and to predict molecular markers associated with fruit quality characteristics. The most preferred molecular markers have been used as single nucleotide polymorphisms (SNPs), polymorphic insertions or deletions (indels), or microsatellites (simple sequence repeats) recently. Genome-wide association studies (GWAS) and linkage mapping need high-throughput molecular marker assays. Although genetic mapping studies are still limited in mandarin due to the insufficiency of phenotype data and the complexity of fruit quality traits, some studies have been conducted on fruit quality traits that are important for Citrus breeding (Şahin-Çevik &

Moore, 2012; Omura & Shimada, 2016; Yu et. al, 2016; Goldenberg et. al, 2018; Imai et. al, 2018). The QTL analysis would help select parents and improve hybrids of the parents with the target gene of a favorable trait by preventing the long juvenile period of Citrus and eliminating Citrus breeding expenses (Asins et al. 2015). Improvements in next-generation sequencing and genotyping array technologies have helped to understand the genetic basis of quantitative trait variation. SNP genotyping became the most widely used genotyping method for GWAS and QTL mapping due to being inexpensive and producing many, codominant SNPs. In addition, SNP genotyping can be performed with SNP arrays or produced by genotyping-by-sequencing (GBS), or whole-genome sequencing.

Fruit size is among the most crucial fruit quality traits for mandarin breeding. Generally, mandarin fruit size varies from small to medium, but tangelo and tangor hybrids have larger fruit sizes. Amparo is a small-fruited mandarin with about 40 mm diameter and 30 g weight. Moreover, Ugly has a much larger fruit size, it is a tangelo with a fruit diameter of 120 mm and a fruit weight of 580 g (Goldenberg et. al, 2018). Obtaining fruits in uniform fruit size is one of the essential elements for mandarin breeding. Therefore, it is important to construct a mandarin genetic map and identify markers related to fruit size. QTL identification associated with fruit size for mandarins has been conducted in several studies (Yu, Chen, and Gmitter 2016; Imai, Yoshioka, and Hayashi 2017). In the study, Fortune (FOR), Murcott (MUR), and 116 F_1 mandarin individuals derived from (Fortune x Murcott) were analyzed for fruit quality traits by Yu et. al (2016). It was carried out in Fortune (FOR), Murcott (MUR), and 116 F_1 mandarin

individuals derived from (Fortune x Murcott) were analyzed for fruit quality traits by Yu et. al (2016). It was carried out in January and February, with four samplings in 2012 and 2013. The map was performed by using a 1536-SNP Illumina GoldenGate assay. The constructed genetic linkage map of “FOR” consisted of 189 SNPs, while "MUR" consisted of 106 SNPs. A total of 48 QTLs related to fruit quality traits were defined in the study. 3 QTLs (FW5.1, FW4.2, and FW8) of them were associated with fruit weight (FW) and 3 QTLs (FD5.1, FD9.3, and FD4.2) of them were related to fruit diameter (FD). The repeatable QTLs were determined as FW5.1 and FW8 and non-repeatable were FW4.2, FD4.2, FD5.1, and FD9.3. FW4.2 was detected on MUR4.2 (Clementine reference scaffold 4) with 25.69 cM and explained % 24.60 of the phenotypic variance (R^2) (Table 1). QTL analysis study on fruit quality characteristics in the mandarins (Imai et. al 2017) detected QTLs associated with fruit size. A SNP-based genetic linkage map and QTL mapping were conducted using an F_1 segregating population derived from (Harehime x Yoshida) by Imai et. al (2017). The map for “Harehime” consisted of 442 SNPs, and for “Yoshida” consisted of 332 SNPs. 4 QTLs (FWq1, FWq2, FWq3, and FWq4) were identified for fruit weight with 14.9-26.5 % of the phenotypic variance. FWq3 (Imai et. al 2017) was identified spanning 15.3-31.0 cM on the Clementine genome scaffold 4. The most striking point in this study is the claim that these two QTLs can correspond since FWq3 (Imai et. al 2017) and FW4.2 (Yu et. al 2016) are located on the same Clementine reference genome scaffold 4 (Table 1).

Table 1. The QTL studies on fruit size in mandarin hybrids

Trait	QTL	Parent	LG	Position (cM)	LOD (threshold)	Phenotypic Variance (%)	Population	Reference
Fruit Weight (FW)	FW4.2	Murcott	MUR4.2	25.7	2.7	24.6	(FOR x MUR) F_1 Population	Yu et. al (2016)
	FW5.1	Fortune	FOR5.1	27.3	2.85	15.03		
	FW5.1	Fortune	FOR5.1	29.3	2.81	24.55		
	FW8	Murcott	MUR8	31.6	2.91	21.2		
	FW8	Murcott	MUR8	29.6	2.76	20.59		
	FW8	Murcott	MUR8	16.6	2.68	20.59		
	FWq1	Harehime	LG3	29.1	3.68	16.7	(Harehime x Yoshida) F_1 Population	Imai et. al (2017)
	FWq2	Harehime	LG3	91.8	3.39	16.4		
	FWq3	Harehime	LG4	31	5.5	26.5		
	FWq3	Harehime	LG4	15.3	3.61	20.2		
	FWq4	Harehime	LG7	20.8	2.75	14.9		
Fruit Diameter (FD)	FD4.2	Murcott	MUR4.2	25.7	2.69	20.5	(FOR x MUR) F_1 Population	Yu et. al (2016)
	FD5.1	Fortune	FOR5.1	31.3	2.9	29.42		
	FD9.3	Fortune	FOR9.3	4.7	2.9	18.61		

The fruit flavor is one of the main determinants of fruit quality and consumer preference in mandarins. Two important factors which affect the taste of fruit are sugar content and acidity (Omura and Shimada 2016; Raveh et al. 2020). The ratio between sugar and acid content, which is defined as the total soluble solids: titratable acidity ratio (TSS/TA), is significant for the taste of the fruit (Goldenberg et al. 2018; Raveh et al. 2020). Only the high sugar content and less acidity rate or less sugar and high acidity level do not affect the fruit taste positively. For this reason, fruit is required to contain a certain amount of acid content in terms of fruit taste. Fruits with a high TSS/TA ratio have a bland taste, while fruits with a low TSS/TA ratio have a sour taste. Goldenberg et. al (2018) stated that the TSS/TA ratio for mandarins that are highly desired is about 13. In addition, Citrus fruit development in relation to solids and acids. Solids gradually increase, and acids first increase and then decrease. So meaningful comparison of varieties is only possible in relation to the date sampled. (Yamasaki et al. 2007; Ye et al. 2009) reported that tangerines, which is a popular variety in terms of their taste, are the variety of mandarin with mean of the highest sugar content (10.92 ^{O}Brix), acid content (0.83), and sugar/acid ratios (13.46) in Table 2. Some QTLs associated with the fruit sugar content in Citrus have been identified in some previous studies. For Soluble Sugar Content (SSC), Yu et. al (2016) detected five non-repeatable (over years) QTLs (SSC2.2, SSC3.2, SSC3.3, SSC4.2, and SSC8) on scaffolds 2,3,4, and 8 of the Clementine reference genomes. In addition, three non-repeatable QTLs (ST1.2, ST7.2, and ST9) for Soluble solids content: titratable acidity (ST) were identified by Yu et. al (2016). Moreover, they identified two non-repeatable QTLs (TA7.1 and TA8) and one repeatable

QTL (TA9) for TA were positioned at scaffolds 7, 8, and 9 of the Clementine reference genome. The QTLs identified for ST were positioned on the scaffold 1, 7, and 9 of the Clementine reference genome (Table 3). In this study, some QTLs for TA overlapped the QTLs for ST and SSC. For example, TA9 and ST9, and TA8 and SSC8 overlapped. Imai et. al (2017) studied one of four mandarin fruit quality traits: sugar content (SC). Only SCq1 was identified for SC on LG5 (on the Clementine genome scaffold 5) (Table 3).

Table 2. The reported mean of sugar content (^{O}Brix), acid content, and sugar/ acid content rates in mandarins by Campbell et al (2008)

Mandarin Varieties	Mean of Sugar Content (OBrix)	Mean of Acid Content	Mean of Sugar/Acid Rate
Satsuma	8.83	0.58	15.96
Clementine	9.3	0.57	17.13
Tangerine	10.92	0.83	13.46

Table 3. The QTL studies on fruit soluble solids and total acids in mandarin hybrids

Trait	QTL	Parent	LG	Position (cM)	LOD (threshold)	Phenotypic Variance (%)	Population	Reference
Soluable Solids Content (SSC)	SSC2.2	Fortune	FOR2.2	35.53	2.92	17.8	(FOR x MUR) F_1 Population	Yu et. al (2016)
	SSC3.2	Murcott	MUR3.2	28.14	2.56	18.42		
	SSC3.3	Fortune	FOR3.3	0	2.89	17.32		
	SSC4.2	Fortune	FOR4.2	18.24	2.92	22.6		
	SSC8	Murcott	MUR8	0	2.73	15.72		
	SCq1	Harehime	LG5	20.6	2.8	14.1	(Harehime x Yoshida) F_1 Population	Imai et. al (2017)
Titratable Acidity (TA)	TA7.1	Fortune	FOR7.1	45.25	2.8	19.8	(FOR x MUR) F_1 Population	Yu et. al (2016)
	TA8	Murcott	MUR8	0	2.99	24.77		
	TA9	Murcott	MUR9	40.65	2.77	20.03		
	TA9	Murcott	MUR9	39.72	2.79	19.33		
SSC/TA	ST1.2	Murcott	MUR1.2	2	2.73	25.3	(FOR x MUR) F_1 Population	Yu et. al (2016)
	ST7.1	Fortune	FOR7.1	45.25	2.85	20.52		
	ST9	Murcott	MUR9	40.65	2.82	17.65		

Seedlessness is a desirable trait for Citrus breeding since the seed in the fruit may negatively affect the taste and aroma of the fruit due to effects of seeds on chemical composition (Yamasaki et. al, 2007; Ye et. al, 2009). Navel orange, Satsuma mandarin, and Clementine mandarin are the most popular Citrus crops that are seedless (Patrickollitrault et al. 2007; Ye et al. 2009; Zhang et al. 2012). For Citrus fruit to be defined as seedless, it must contain no seeds, contain aborted seeds, or the number of seeds of a multi-seeded variety must be significantly reduced (Vardi, Levin, and Carmi 2008; Goldenberg et al. 2018). Many factors play a role in mandarin seedlessness, including parthenocarpy, male and female sterility, self-incompatibility, abnormal ovules, embryo sac abortion, environmental conditions, and plant growth regulators (Zhang et. al, 2012; Goldenberg et. al, 2018). Seedlessness is one of the most important fruit quality characteristics in mandarin breeding and is important for obtaining seedless fruits. Thus, QTLs responsible for this trait are required to select for it more effectively. Five genomic regions were detected for seed number by (Asins et. al, 2015) using 201 full-sib population, which were crossed reciprocally between Fortune (Cl) and Chandler (Gr). Four QTLs associated with seed numbers were detected in 2014 and 2015. The QTL SN11 was identified on the linkage group Gr9b (Chandler) on locus CTUCH7 with 8.8 %, moreover the other QTLs on the Clementine linkage groups (Cl7a, Cl7c, and Cl10) with 8.4, 10.7, and 11.3 % of the explained phenotypic variance, respectively. Yu et. al (2016) detected non-repeatable QTLs (SD3.1 and SD9.1) associated with seed number. These QTLs on scaffolds 3 and 9 of the Clementine reference genome explained phenotypic variance with 21.32% and 19.59 %, respectively (Table 4).

Table 4. The QTL studies on seed number in mandarin hybrids

Trait	QTL	Parent	LG	Position (cM)	LOD (threshold)	Phenotypic Variance (%)	Population	Reference
Seed Number (SN or SD)	SN11	Chandler	Gr9b	34.600	1.98	8.8	Fortune x Chandler	Asins et. al (2015)
	SN12	Fortune	Cl7a	51.952	2.41	8.4		
	SN14	Fortune	Cl7c	0	3.38	10.7		
	SN14	Fortune	Cl10	119.400	3.04	11.3		
	SD3.1	Fortune	FOR3.1	11.09	2.94	21.32	(FOR x MUR) F_1 Population	Yu et. al (2016)
	SD9.1	Fortune	FOR9.1	9.2	2.89	19.59		

Fruit color is one of the most influential fruit quality attributes of Citrus features because the first thing that affects consumer preference is the appearance and color of the fruit (Goldenberg et. al, 2018). Citrus peels consist of the pigmented peripheral epicarp or flavedo (colorful part) and albedo (colorless part or white part) (Figure 1, (Matheyambath, Padmanabhan, and Paliyath 2016; Caputo et al. 2020).

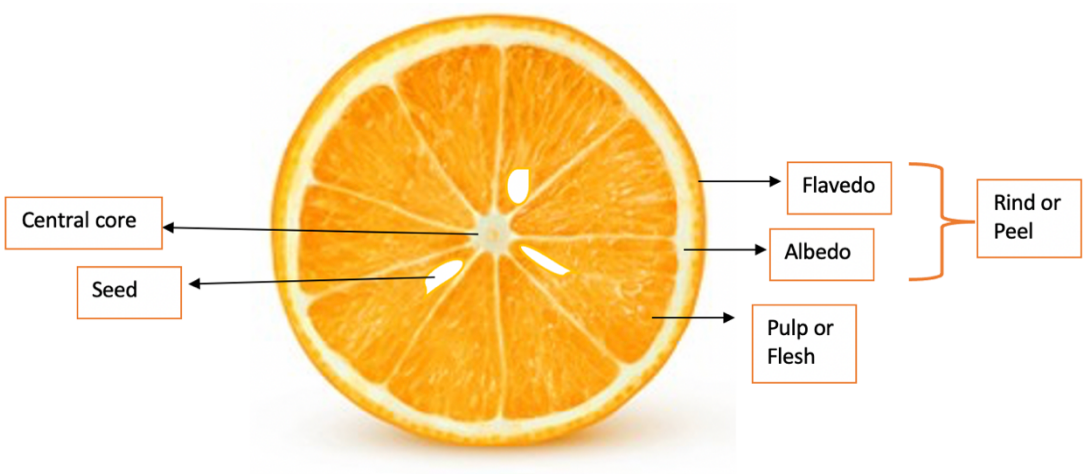


Figure 1. A schematic fruit section of typical Citrus fruit. Inspired by (Liu, Heying, and Tanumihardjo 2012; Putnik et al. 2017; Katz et al. 2007; Sadka et al. 2019)

The part responsible for the fruit peel color is the flavedo (outer part) of the fruit (Matheyambath et. al, 2016). The color of mandarin peels is usually greenish-yellow, yellow, yellow-orange, orange, and reddish. Fruit pulp color is as important as a fruit quality feature as peel color. Color pigments are responsible for the color of fruit flesh and skin. These pigments are carotenoids and flavonoids. In general, mandarins have a yellow to orange hue. Carotenoids are the primary pigments for yellow to orange colors.

There are three main cultivar groups in terms of including carotenoids in Citrus. The first group is the carotenoid-poor group. There are pomelos, lemons and limes, and grapefruits in this group. The other group is the violaxanthin-abundant group, which includes oranges. The third is the β-cryptoxanthin-abundant group, primarily containing mandarins (Goldenberg et. al, 2018). Lycopene, which is responsible for the pink color, is also a carotene and is found in grapefruit such as 'Star RUBY' and navel orange such as 'Cara Cara.' Anthocyanins are a subgroup of flavonoids that give blue, purple, and red colors to the fruit. Anthocyanins are phenolic compounds responsible for the red color of Citrus fruits such as blood oranges (eg'Moro,' 'Taracco' and 'Sanguinello'). In addition, with the increase in the popularity of red-colored fruits, mandarin hybrids with red flesh have been introduced to the global market. These red-fleshed mandarin hybrids are rich in anthocyanins, and examples include 'Sun Red,' 'Early Sicily,' and 'Sweet Sicily' varieties released in Italy (Crifò et al. 2012; Chen, Lo Piero, and Gmitter 2015; Goldenberg et al. 2018; Zhu et al. 2020). There are some significant genes in the carotenoid biosynthesis pathway in Citrus. They are PSY, PDS, LCYB1, LCYB2, CHY, CHYB, and CCD. The gene responsible for anthocyanin biosynthesis in Citrus is the *RUBY* gene which encodes an MYB transcription factor. MYB transcription factor regulates anthocyanin biosynthesis with a basic helix-loop-helix (bHLH) transcription factor and a WD40 repeat protein. The expression of the RUBY gene depends on environmental conditions. This gene is upregulated under cold temperatures, causing anthocyanin accumulation in the fruit (Butelli et al. 2012, 2017; Crifò et al. 2012; Huang et al. 2019; Sicilia et al. 2020)

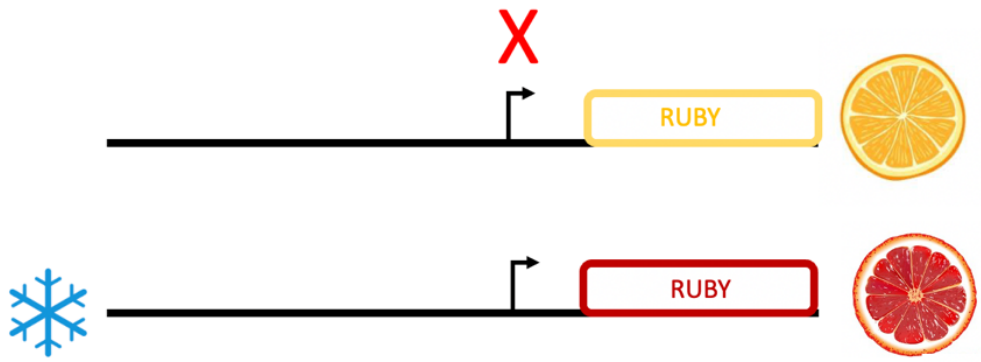


Figure 2. RUBY gene regulation under cold temperatures in blood orange. Inspired by (Lisch, 2013; Butelli et. al, 2017)

Previous QTL studies on fruit color were based on detecting QTLs associated with carotene content in Citrus fruits. Papers reporting QTLs associated with anthocyanin content in Citrus have not yet been published. (Sugiyama et al. 2011) created a population (AG) derived from the female parent 'Okitsu-46' (A255) and the male parent 'Nou-5' (G434). For QTL mapping, EST-based CAPS markers were used to generate linkage maps from 51 progenies and their parents. Their extracts were prepared to measure by HPLC the carotenoid content. According to the results, the A255 map was generated with 345 markers and covered 660cM. In contrast, the G434 map was constructed with 254 markers covering 642 cM. It was cited that there was transgressive segregation for total and each carotenoid contents in progenies. Transgressive segregation arises from the distribution of alleles between parents. It leads to the form of extreme phenotypes in segregated populations compared to the parental phenotypes. For plant breeding, including Citrus breeding, it is important that the traits show transgressive

segregation to identify improved new hybrids relative to their progenitors (V, Nirubana et al. 2021) 40 QTLs for β-cryptoxanthin, total carotenoids, and other carotenoid content were identified. 13 QTLs are associated with total carotenoids among all loci of the markers in linkage groups. The QTLs associated with β-cryptoxanthin, total carotenoids, and other, carotenoid content (carotene 1, carotene 2, carotene 3, lutein, total violaxanthin, 9-cis-violaxanthin, all-trans-violaxanthin, phytoene) were overlapped on A-07, G-06, and G-07. In this study, two QTLs with the highest scores were pointed out, one on LG6 on the 'Nou-5' map at GN0005 and the other on LG7 on the Okitsu-46 map Bf0136 (Table 5). However, it was noted that both QTLs contributed negatively to the total carotenoid content. Asins et. al (2015) reported that QTLs were detected for fruit color(cF) and juice color (cJ) in the Fortune (Cl) x Chandler (Gr) full-sib population. cF_12, cF_14, cF_12, and cJ_14 were located on Clementine map (Cl10) with 11.7%, 16.4 %, 7.9%, and 11.4 % of explained phenotypic variance, respectively. On the other hand, the QTLs for cF_12, cF_14, cF_12, and cJ_14 were detected on the Chandler map (Gr10 and Gr4a). cJ_12 on Gr10 explained the highest variance, 46.1%, among the other QTLs for fruit color (Table 5). Yu et. al (2016) reported that 28 QTLs were detected in the Fortune x Murcott population for juice and flavedo (peel) color. FCL4.1, FCA4.1, FCB4.1, FCAB4.1, JCA4.1, and JCAB4.1 on MUR 4.1 (on scaffold 4 of the Clementine reference genome) overlapped. It explained significant variance in both years with 19.35% and 14.44% of phenotypic variance, respectively. Overall, the QTLs associated with carotenoid content for juice and peel color were positioned on scaffolds 1, 4, 7, and 8 of the Clementine reference genome (Table 5). 138 putative QTLs for 57 flavonoids

were reported in an F_1 pseudo-cross population derived from *Citrus reticulata* x *Poncirus trifoliata* by (Mou et al. 2021). Four tissues sampled, young and old leaf, mature fruit pericarp, and fruit pulp, were used for the analysis by constructing a SNP-based high density genetic map. Metabolite and expression quantitative trait locus (mQTL and eQTL) were performed for QTL analysis. 25 QTLs for fruit pericarp and 35 QTLs for pulp were identified. Each QTL for flavonoids in fruit pericarp was explained by phenotypic variance from 30.2 to 71.8%. At the same time, each QTL for flavonoids in fruit pericarp was explained by phenotypic variance from 26.7 to 63.0%. The QTLs for flavonoids were on Chr3, Chr7, and Chr9.

Table 5. The QTL studies on fruit color in mandarin hybrids

Trait	QTL	Parent	LG	Position (cM)	LOD (threshold)	Phenotypic Variance (%)	Population	Reference
Fruit Color	The QTLs for Caretenoids	Okitsu	A07	37.3	1.1-1.7	9.6-14.4	Okitsu (A255) x Nou-5 (G-434)	Sugiyama et. al (2011)
	The QTLs for Caretenoids	Nou-5	G06	7	1.6-3.4	13.3-26.9		
	The QTLs for Caretenoids	Nou-5	G07	66.3	1.3-2.0	11.3-17.0		
	cJ14	Fortune	Cl10	26.618	3.63	11.7	Fortune x Chandler	Asins et. al (2015)
	cJ12	Fortune	Cl10	46.1	5.64	16.4		
	cF12	Fortune	Cl10	31.618	2.25	7.9		
	cF14	Fortune	Cl10	37.8	2.68	11.4		
	FCL4.1	Murcott	MUR4.1	1.82	2.58	40.33	(FOR x MUR) F_1 Population	Yu et. al (2016)
	FCA4.1	Murcott	MUR4.1	2.66	2.66	49.43		
	FCA8	Murcott	MUR8	2.69	2.69	19.35		
	FCB4.1	Murcott	MUR4.1	2.5	2.5	30.7		
	FCAB4.1	Murcott	MUR4.1	2.68	2.68	42.26		
	JCA4.1	Murcott	MUR4.1	2.61	2.61	31.14		
	JCAB4.1	Murcott	MUR4.1	2.65	2.65	19.81		

Since it is directly related to the external appearance, the **rind** or **peel thickness** is a vital fruit quality feature. The peel or rind of the fruit has flavedo and albedo sections and it is reported (Ladaniya 2010; Cronjé, Zacarías, and Alférez 2017) that mandarins have thinner albedo thickness among other Citrus varieties in general. For example, the albedo thickness of sweet oranges varies from 5 mm to 10 mm. Grapefruits have an albedo thickness of more than 10 mm. Lemons have ±5 mm, and mandarins have an albedo thickness of less than 3 mm on average. Asins et. al (2015) performed a QTL analysis associated with rind thickness. A total of QTLs for rind thickness was on the Clementine linkage group, with phenotypic variation explained by each QTL ranging from 9.0 to 21.3% (Table 6).

The **juice volume** is an essential trait for inner fruit quality. Pulp segments filled with many vehicles are called juice sacs in the Citrus flavedo part. Juice sacs include sugars, organic acids, vitamins, and polyphenolic plant compounds. Juice volume is considered an important criterion, especially for the fruit juice industry (Kimball 1999; Katz et al. 2007). 2 QTLs for juice volume were detected on Clementine linkage groups by Asins et. al (2015). The QTL JV_11 on Cl3 explained 7.4% of phenotypic variance, and JV_11 on Cl4b explained 10.8% of phenotypic variance. In addition, 7 QTLs were identified for juice content (JC). The paper reported that JC was calculated as a percentage of JV and fruit weight (FW). All QTLs related to juice volume and content are defined only on the Clementine map (Table 7). Yu et. al (2016) identified only one QTL associated with juice percentage (JP). JP was calculated by using juice volume and fruit

weight. The QTL JP7.2 was on MUR 7.2 (on the Clementine genome scaffold 7) with a 2.78 LOD score (Table 7).

In this study, the parents of the mandarin hybrids differ in several fruit traits related to size, seediness, sugar content, and acidity. We sought to achieve a better understanding of the genetic basis of variation in fruit characteristics, including fruit quality and rind color in an outcross F_1 population of mandarin hybrids. The population has phenotypic diversity for each trait. We will compare the QTLs detected in prior studies to the QTLs detected in this study. For the work, a large number of high-quality SNP markers, the use of high-throughput phenotyping, and a parent with blood orange in the pedigree were used. A unique aspect of this research is the possible detection of QTLs associated with red color in the peel derived from the blood orange grandparent.

Table 6. The QTL studies on rind thickness in Citrus

Trait	QTL	Parent	LG	Position (cM)	LOD (threshold)	Phenotypic Variance (%)	Population	Reference
Rind Thickness (RT)	RT_11	Fortune	Cl7a	32.948	5.24	15.6	Fortune x Chandler	Asins et. al (2015)
	RT_12	Fortune	Cl3	47.158	2.61	9		
	RT_12	Fortune	Cl7a	32.948	6.61	21.3		
	RT_14	Fortune	Cl4b	5	3.5	13.2		
	RT_14	Fortune	Cl7a	32.9	7.23	20.8		

Table 7. The QTL studies on juice volume in Citrus

Trait	QTL	Parent	LG	Position (cM)	LOD (threshold)	Phenotypic Variance (%)	Population	Reference
Juice Volume (JV or JP)	JV_11	Fortune	Cl3	32.948	5.24	15.6	Fortune x Chandler	Asins et. al (2015)
	JV_11	Fortune	Cl4b	47.158	2.61	9		
	JP7.2	Murcott	MUR7.2	0	2.78	24.6	(FOR x MUR) F_1 Population	Yu et. al (2016)

MATERIALS and METHODS

PLANT MATERIALS

159 trees were grown for this project in the University of California Riverside (UCR) citrus fields, but since 59 trees were removed, the study continued with 100 trees. Phenotypic and genotypic analyzes were performed on the remaining trees. Since some individual trees on which DNA extraction was performed did not have any fruit, fruit quality characteristics could not be measured, and therefore, the study was continued with a total of 93 individuals.

METHODS

OVERVIEW of the MAPPING POPULATION

The parents of this population differ in their ability to accumulate anthocyanins or carotenoids. Kiyomi is a typically colored mandarin due to yellow and orange carotenoids (Figure 3). Amoa 8, on the other hand, is an anthocyanin-rich hybrid between Moro blood orange and Avana mandarin ("UCR: Citrus Variety Collection" n.d.) (Figure 3). Owing to its blood orange parentage, Amoa 8 accumulates anthocyanins in the fruit flesh and peel, giving it a deep red coloration. The F_1 intercross progenies exhibit variation in anthocyanins and carotenoids accumulation and fruit quality traits (Figure 4).

The pollinations were performed on April 11, 2011, using Kiyomi as the female parent and Amoa 8 as the male. 18 pollinations were made. This resulted in 10 fruit that set seed. These 10 fruit produced 232 seeds that were planted in seed cones in early 2012. Approximately 175 seeds germinated and were grafted by Spring 2013. 131 grafted trees were planted in Field 11B (the location in UCR fields) on June 24, 2014, and 39 grafted trees were planted in Field 12C (the location in UCR fields) on July 9, 2015. Trees that were planted in 2015 were held back because they were too small to plant the prior year or had to be repropagated. Any grafted trees not planted were likely discarded because they failed to thrive. Some of the trees in 11B began to set fruit as early as 2016. Both Carrizo and C-35 citranges were used as rootstocks. These trees were about 6 years old when the phenotyping was done.

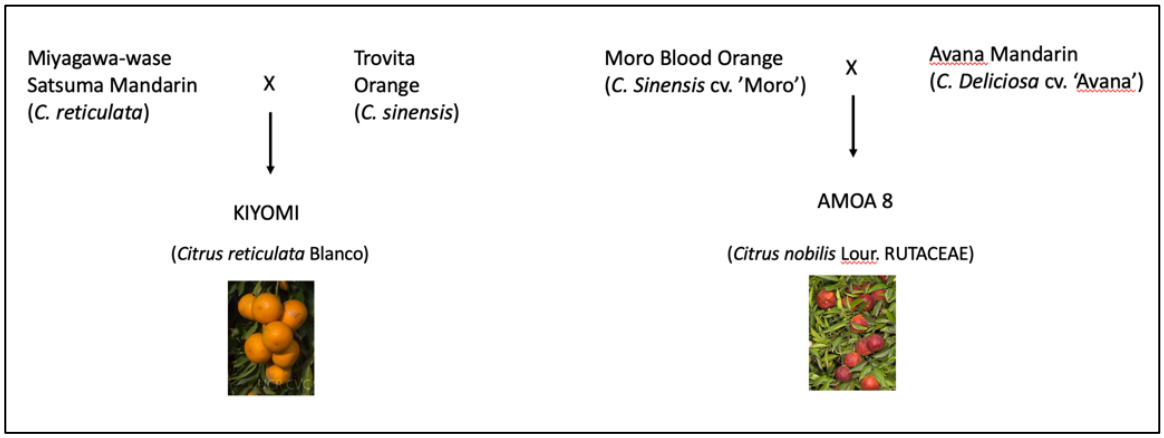


Figure 3. The Parents of the population

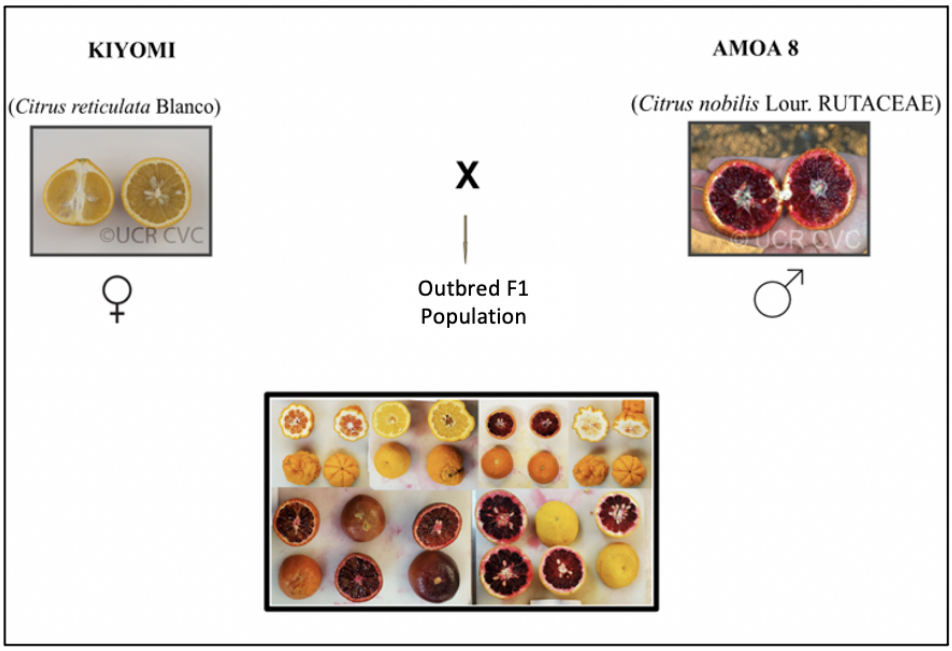


Figure 4. The overview of outbred F_1 Population

TRAIT EVALUATION

Fruits were collected from 159 Kiyomi x Amoa 8 hybrids at the UCR Citrus fields during the 2019/2020 season in December to identify loci of interest that could be contributing to variation in fruit quality traits for QTL mapping. First, the identities of the trees were written on each bag, and then the fruits were picked according to their tree identities. 50 fruits per tree were collected in the labeled bags for phenotypic evaluation. Before being sent for phenotypic analysis, each bag was washed in detergent water, rinsed, and left to dry under sunlight to reduce the risk of HLB contamination (Figure 5). Each bag was checked for the presence of materials that may carry a contamination risk, such as leaves or stems, which were then removed. All fruit bags were then transported

for analysis of phenotypic features to the UC Lindcove Research & Extension Center (LREC) (Figure 5).

Figure 5. Fruits prepared for phenotypic analysis (a), Packline data measurement in LREC (b), and (c)

In LREC, the phenotypic features were evaluated as packline and destructive features (Table 1). The packline data was evaluated by grading the fruits by two distinct Compac® software programs with Sizer® and InVision®.

Table 8. The list of the destructive and packline traits

Destructive Traits	Packline Traits		Peel Color
Average seed number	Fruit weight	Stem size	Moro red
Average peel thickness	Fruit volume	Calyx size	Red
Soluble sugar content	Major diameter	Flatness	Red orange
Juice weight	Minor diameter	Texture	Dark orange
Juice volume	Elongation	Smoothness	Orange
Titratable acidity	Overall Roundness	Rough skin	Orange yellow
pH	Stem angle	Smooth skin	Yellow
Acid content		Peel Color	Yellow green
			Green
			Dark green

Sizer® software evaluated the **fruit weight** and **volume** of the fruits from the packline samples. Fruits were passed over a weighbridge to measure fruit weight in grams (g) and then fruit weight measurement values were sent to Sizer®. **Fruit volume** was measured as displacement in cubic centimeters (cm^3) and converted to milliliters (mL) by using fruit diameter. Fruit volume values were sent to Sizer® and In Vision® software and then they were evaluated by the two software.

The other packline traits were measured by InVision® software. For the measurement of fruit diameter, which is related to fruit size, two different measurements, defined as major and minor diameter, were completed. The diameter of fruit at the equator in millimeters (mm) was measured for **major diameter** (Figure 6 (a)). For **minor diameter**, the diameter of fruit in any direction in millimeters (mm) was evaluated (Figure 6 (b)).

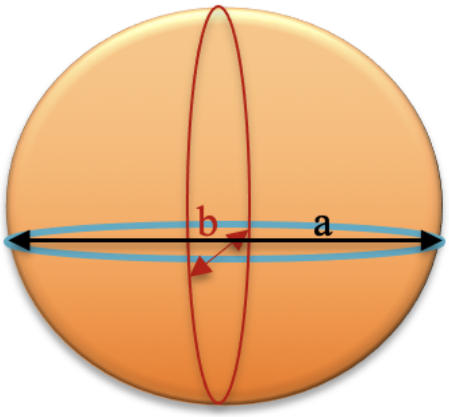


Figure 6. Major (a) and minor (b) diameter measurements of fruit

Elongation measurements of fruits were also determined by InVision® software. It was given values from 0 to 100, where 0 was referred to as a sphere and 100 was referred to as a rod. **Overall roundness** measurement is similar to elongation measurement. However, 0 value was a rod and 100 value was a sphere for overall roundness. **Stem angle** was measured as the angle between the stem and blossom ends (calyx) by InVision®**. Fruit stem size** was expressed in millimeters by measuring the diameter of the fruit stem. **Fruit calyx size** was determined in millimeters by measuring the diameter of the fruit blossom ends (Figure 7). **Flatness** was measured, which allows the detection of sunburn or other flat spots, where 0 value was a perfect sphere and 100 value was a flat board. The **texture** is a trait that was measured around the fruit with rough skin in square millimeters (mm^2). The Texture data report the values for smooth skin, rough skin, ridge, and crease. Meanwhile, texture areas are affected by the smoothness and rough skin. The InVision® software captures approximately 30 images of each piece of fruit

from a lot of angles since the fruit rotates as the belt moves along during fruit grading process. It is developed by selecting individual fruit pixels that reveal a shadow, caused by many types of blemishes. A seed is planted by selecting a pixel that has the shadow and defining that as "rough texture". Likewise, pixels are selected for "smooth texture". Thus, according to shadows texture areas' **smoothness** or **rough skin** were determined. The InVision software calculates the total area of shadow and reports this value.

Figure 7. Stem angle (a), calyx size (b), stem size (c) measurements area highlighted by red

The other packline trait is the **fruit color** measured by InVision® software. For each pixel, they assign it to one of ten color categories. They do this for all pixels in the image. They assign each image a value for each of the ten colors. The value is the percentage of pixels (per fruit) that are a certain color. Therefore, each fruit has a value for the color. Colors were defined and referenced from color sample standards from *The Royal Horticultural Society's Colour Chart*. Each color sample was matched to actual fruit colors chosen according to the color chart used by the UC research community.

From the 50 fruit per tree analyzed using the packline, 12 fruit were randomly selected for destructive sampling. Then, these twelve fruits per tree were destructively sampled to obtain measurements for the additional traits. The fruits were prepared for destructive data analysis by cutting them in half at the equatorial region.

The number of seeds for each fruit of twelve fruits per tree was counted as the number of seeds visible in an equatorial cut. Then, the total number of seeds of these twelve fruits was divided by the number of twelve fruits and the **average seeds number** was calculated.

Fruit peel thickness was determined by measuring the flavedo portion of twelve fruits per tree by a digital caliper and then the values were transferred to the computer automatically. **Average peel thickness** data was obtained by dividing the total peel thickness value per fruit by the total fruit number.

After measuring the seed and peel thickness of the fruits, a hydraulic fruit press was used to extract fruit juice for further measurements. For each tree, juice from twelve fruit was combined. Then, the **juice weight** of each juice sample was measured with a precision balance and expressed in grams. **Juice volume** was calculated using a graduated cylinder.

The trait specified as the **sugar content** is the soluble solids content of the fruit. ^{O}Brix value was obtained by measuring the juice from twelve fruits per tree using a refractometer (Bellingham + Stanley RFM 110 Refractometer). The total ^{O}Brix values per tree were divided by the total number of fruits and the sugar ratios of the tree identities were calculated as ^{O}Brix.

Titratable acidity (TA), pH, and acid content are features that are related to each other. The pH of juice is a measure of the concentration of free hydrogen ions in solution. The juice was diluted with water and then titrated. The abbreviations of all measured fruit quality characteristics are given in Table 8.

For the **flesh color,** the pictures of the cut fruits were taken with a custom imaging cabinet. Fruit flesh color was evaluated according to the degree of red color. In the measurement of fruit flesh color, values from 1 to 5 were given according to the redness of the fruit flesh. According to these values, 1 was referred to as non-red colored fruits, while 5 was evaluated as the fruits with the darkest red-colored flesh (Figure 8). The abbreviations of all measured fruit quality characteristics were given in Table 9.

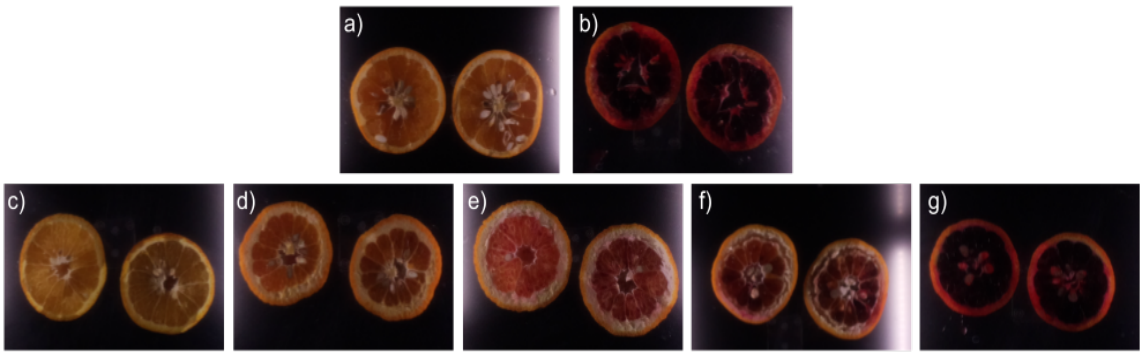


Figure 8. Fruit flesh red color scale: Kiyomi (a), Amoa 8 (b), non-red colored fruit,1 (c), 2 (d), 3 (e), 4 (f), and the darkest red colored fruit, 5 (g)

Table 9. The abbreviation of the fruit traits

Abbreviation	Fruit Trait	Abbreviation	Fruit Trait	Abbreviation	Fruit Trait
JW	Juice Weight	**ELG**	Elongation	**CMR**	Color Moro Red
JV	Juice Volume	**TEX**	Texture	**CR**	Color Red
TA	Titratable Acidity	**OVR**	Overall Roundness	**CRO**	Color Red Orange
Ph	Potential of Hydrogen	**FLT**	Flatness	**CDO**	Color Dark Orange
SC	Sugar Content	**STA**	Stem Angle	**CO**	Color Orange
AC	Acid Content	**STS**	Stem Size	**COY**	Color Orange Yellow
ASN	Average Seed Number	**CAS**	Calyx Size	**CY**	Color Yellow
APT	Average Peel Thickness	**SMT**	Smoothness	**CYG**	Color Yellow Green
FW	Fruit Weight	**RS**	Rough Skin	**CG**	Color Green
FV	Fruit Volume	**SS**	Smooth Skin	**CDG**	Color Dark Green
MajorFD	Major Diameter	**FC**	Flesh Color		
MinorFD	Minor Diameter				

DESCRIPTIVE STATISTICAL ANALYSIS

For Packline data analysis, an average of 50 fruits were analyzed. Then, 12 fruits were selected randomly from 50 fruit bags for destructive data analysis, and the destructive analysis was completed. The data of the fruits whose analysis was completed were submitted as two separate files. After taking the averages of each fruit, both data files were merged according to tree identities to create a phenotype file. The data were transformed using the bestNormalize package in R Studio Software for each of the fruit characters. The bestNormalize package determined which method could be performed to normalize data for each trait. The distribution of the transformed data was examined. In addition, Spearman correlation coefficients were performed to find the relationship among the phenotypic traits of the fruits. The correlation was visualized using the R package corrplot (Simko 2021).

GENOMIC DNA EXTRACTION AND SNP GENOTYPING

Genomic DNA samples were extracted from fresh leaves of mandarin hybrids using the Cetyltrimethyl ammonium bromide (CTAB) protocol with the General 96 Well-Plate CTAB DNA Extraction method. DNA samples were sent to Affymetrix for genotyping using the Citrus Axiom Genotyping 50 K Array in August 2021. All SNP probes were designed relative to the Clementine genome V1.0 (http://www.phytozome.net). The physical position of each SNP corresponding to the Clementine genome was obtained. The genotyping data was received in January 2022. 50064 SNP markers were genotyped and a total of the 20035 SNP markers segregated

between the two parents and were used for QTL mapping (Broman 2009; Broman and Sen 2011).

LINKAGE ANALYSIS and MAP CONSTRUCTION

Linkage analysis was conducted using a pseudo-test cross strategy in the R/qtl package. SNPs were divided into three categories according to their segregation patterns: AB × AA (1:1 segregation only in Kiyomi (maternal parent)), AA × AB (1:1 segregation only in Amoa 8 (paternal parent)), and AB × AB (1:2:1 segregation in both parents).

Although some individuals were sampled for DNA extraction, they could not be analyzed for phenotype data because they did not have any fruit. For this reason, the individuals were filtered to keep the ones with both phenotype and genotype data. There were 93 samples with complete genotype and phenotype data for QTL analysis. To perform QTL mapping, the R/qtl package was used to build maternal and paternal genetic maps and identify QTL. A total of 4491 markers are informative for mapping QTL derived from Amoa 8 (i.e. homozygous in Kiyomi and heterozygous in Amoa 8) and 7303 markers are informative for mapping QTL derived from Kiyomi (homozygous in Amoa 8, heterozygous in Kiyomi). The recommended quality control procedures were followed according to (Broman 2009; Broman and Sen 2011) to filter markers prior to QTL mapping. This includes removing distorted markers, identifying markers incorrectly placed, removing duplicated and switched markers, estimating the recombination fraction between them, calculating a LOD score for the test of $r = 0.5$ for each pair of markers, and counting crossing over numbers. SNPs with any missing data were removed.

RESULTS

ANALYSIS OF PHENOTYPIC TRAIT DISTRIBUTIONS

Fruit quality traits have a significant effect on consumers in the global industry. Identifying the genetic bases of important fruit quality traits is essential for Citrus breeding. Understanding how mandarin fruit quality traits are genetically regulated and correlated is the first step toward improving marker-assisted breeding programs. The distribution of the offspring and the parents of the population in terms of fruit quality characteristics measured was examined as well as the correlation between these features.

The fruit quality traits were analyzed for Kiyomi, Amoa 8, and their outcross F_1 mandarin progenies. The phenotypic trait distributions of the parents and progenies were shown as non-transformed (Raw data) in Figure A1, A2, and transformed data (Transformed data) in Fig. 9, Fig. 10, Fig. 11, and Figure A3. The distributions were transformed using the Best-normalization package in R, and the best transformation method for each phenotypic trait (Figure B2). Trait distributions were typically uni-modal with transgressive segregation evident in most of the measured traits. Transformed phenotypic distributions of destructive traits, which were JW, JV, TA, pH, SC, AC, ASN, and APT, in the populations, transgress beyond the two parents (Figure 9). For example, the average sugar content from 12 fruits of Amoa 8 was 14.6 ^{o}Brix while that of Kiyomi was 11.9 ^{O}Brix. The minimum (min) and maximum (max) values of hybrids are 10 and 17.8 ^{O}Brix, respectively. The value of Amoa 8 was measured as 3.62 mm and Kiyomi was 4.57 mm for APT (Figure B1). The TA, AC, and pH values, which were responsible

for the acidity of the fruit, and TA, AC, and pH values of the parents were closer to each other.

FW, FV, MajorFD, and MinorFD are traits related to fruit size. Looking at the distribution histograms of these features, it is seen that these features were transgressively segregated beyond two parents (Figure 10). MajorFD is the measurement of the average diameter of the fruits from the equatorial region. Amoa 8's MajorFD measured 37.9 cm and 68.58 cm for Kiyomi. The min value for MajorFD was measured as 1.77 cm and the max value was 84.21 cm in the population (Figure B1). The distributions of fruit characters, which are ELG, TEX, OVR, FLT, STA, STS, CAS, SMT, RS, and SS that contribute to the difference in fruit shape and size were also examined (Figure A1, 2). In addition, it is seen that the frequency distributions of the hybrids for these fruit characteristics were often substantially broader than those of their parents (Figure A3).

The progenies and the parents were also evaluated for fruit color traits. The distribution of ten features, CMR, CR, CRO, CDO, CO, COY, CY, CYG, CG, and CDG, related to fruit color, which is one of the most important fruit quality traits related to the external appearance of the fruit, was shown in Figure 11. Transgressive segregation was observed in all the color traits, but to varying degrees. For CMR, CRO, CG, and CYG distributions, parental values span the range of progeny values with less transgression. Most progeny values were similar to parental values in CMR, CRO, CG, and CYG distributions. On the other hand, parental values are similar with extensive transgression in progenies in the distribution of CR, CDO, COY, and CY traits. Since the value of Kiyomi was very low, it was not seen in the CDG distribution histogram.

In the fruit flesh color measurement evaluated according to the red color intensity, the distribution of the offspring and their parents was examined. It was obvious that most individuals do not have red-colored flesh (Figure 12).

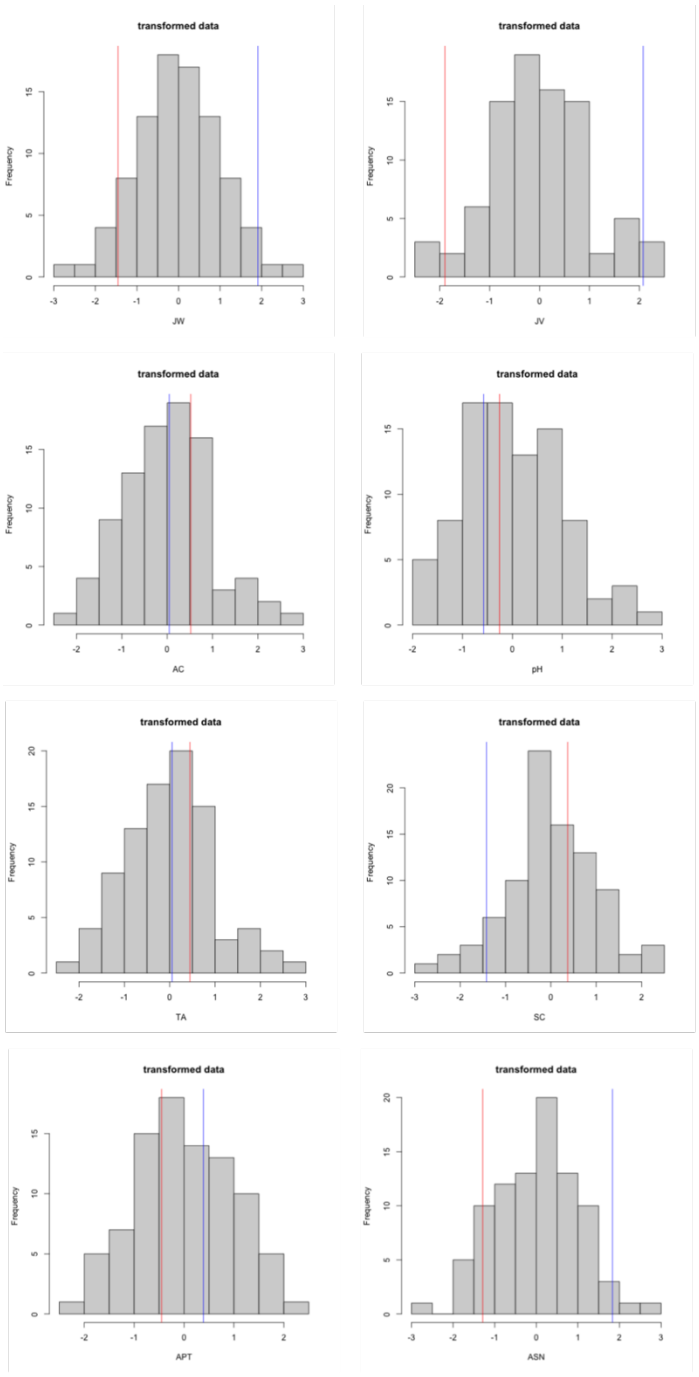


Figure 9. Histograms depict the distribution of destructive traits. The parents Kiyomi and Amoa 8 were represented by the blue and red color lines, respectively

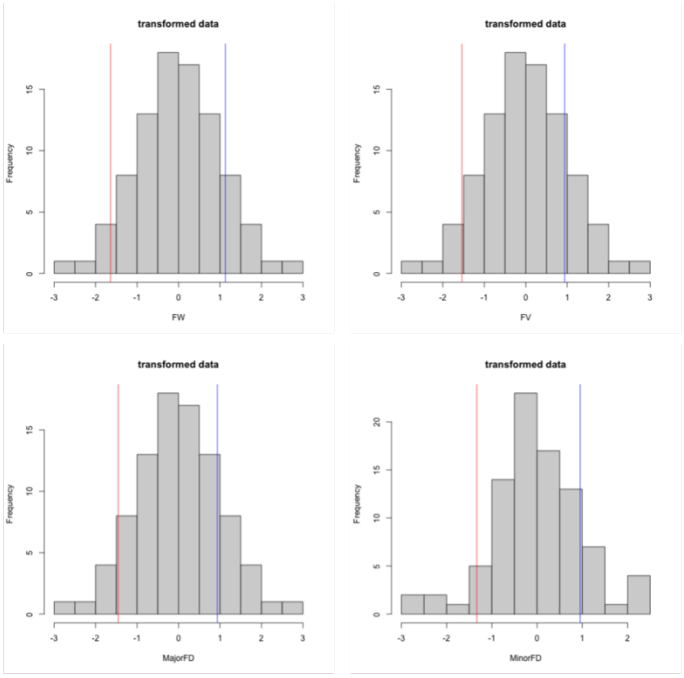
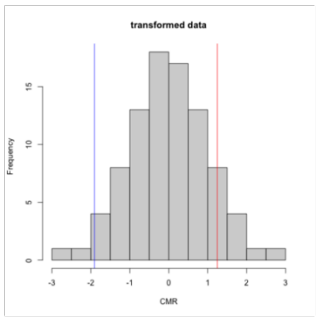
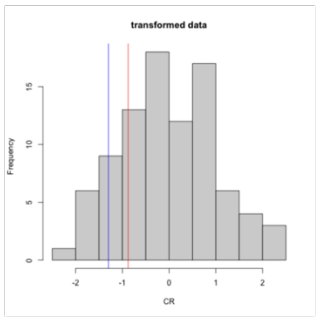
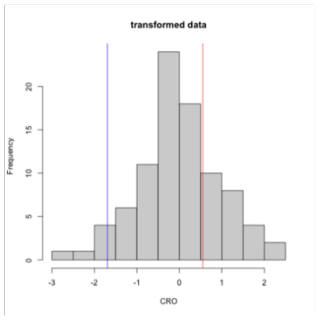
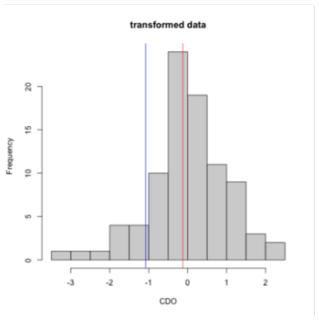


Figure 10. Distribution histograms of FW, FV, MajorFD, and MinorFD related to fruit size. The parents Kiyomi and Amoa 8 were represented by the blue and red color lines, respectively


transformed data
Frequency
CMR


transformed data
Frequency
CR


transformed data
Frequency
CRO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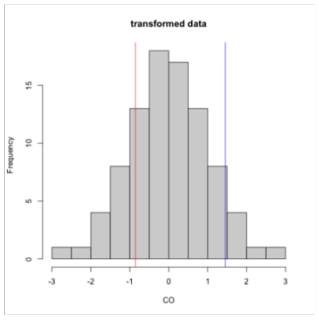
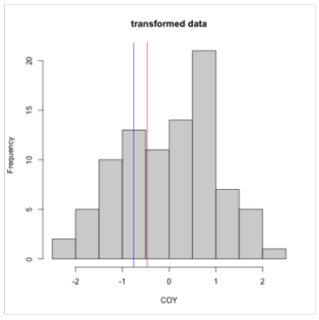
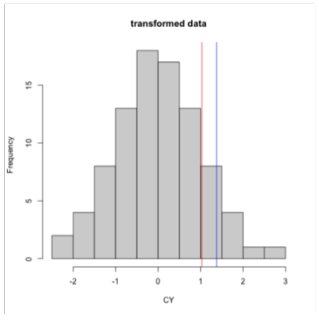
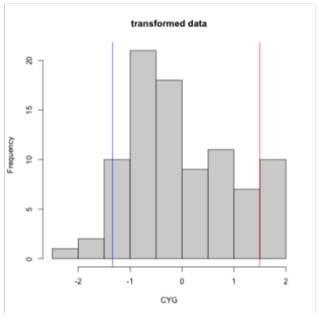
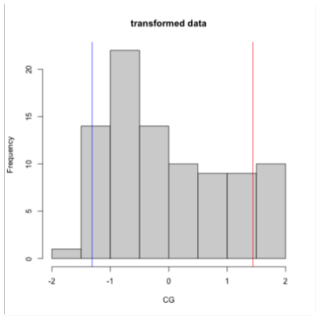
Frequency
CDO


transformed data
Frequency
CO


transformed data
Frequency
COY


transformed data
Frequency
CY


transformed data
Frequency
CYG


transformed data
Frequency
CG

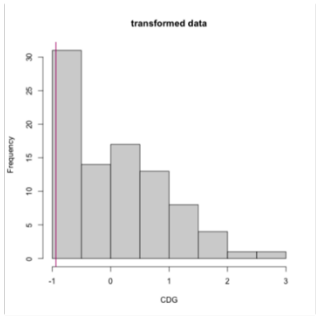

transformed data
Frequency
CDG

Figure 11. Distribution transformed histograms of CMR, CR, CRO, CDO, CO, COY, CY, CYG, CG, and CDG related to fruit color. The parents Kiyomi and Amoa 8 were represented by the blue and red color lines, respectively.

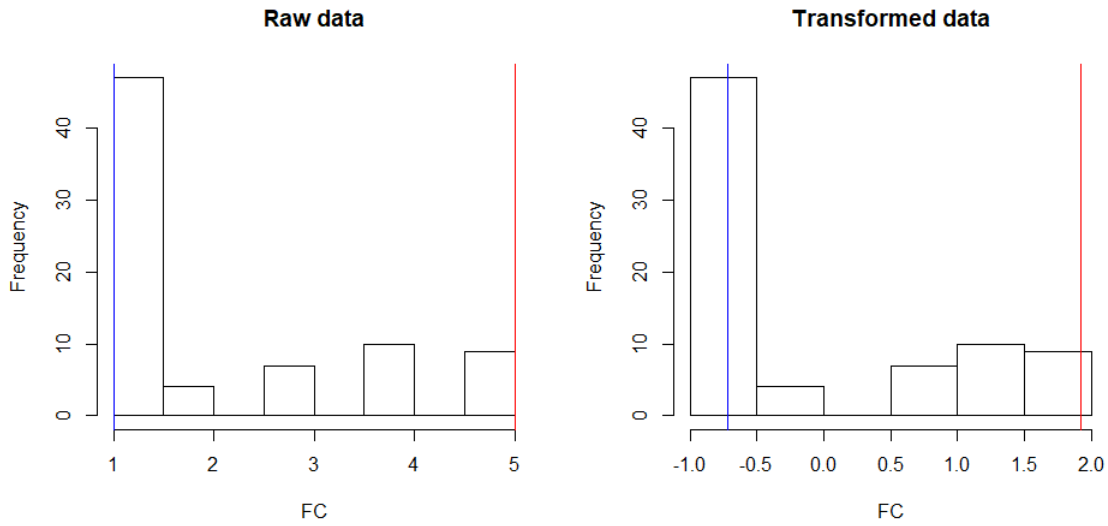


Figure 12. Distribution raw and transformed histograms of FC trait. The parents Kiyomi and Amoa 8 were represented by the blue and red color lines, respectively

CORRELATION ANALYSIS OF PHENOTYPIC TRAITS

Correlations between fruit quality characteristics were evaluated and the high correlations between certain phenotypic traits demonstrated that the phenotypic data were quite consistent. For example, it is expected that the correlations between the characteristics related to fruit size are high. Because fruit weight and diameter are linearly related to each other.

The Spearman correlation analysis was performed for all phenotypic traits. The correlations between the fruit quality traits and fruit characteristics were indicated in Figure 13. The correlations were classified as high if $r > 0.7$, moderate if $0.4 < r < 0.7$ and weak if $r < 0.4$.

According to the correlations of destructive data traits, it is seen that there was a high correlation (r= 0.99) between JW and JV. TA and AC, which are the traits related to fruit acidity, exhibited a strong positive correlation (r= 0.99) whereas there were negative correlations (r= -0.88) between pH and TA and AC. The traits related to fruit size (FW, FV, MajorFD, MinorFD) are highly positively correlated with each other. TEX, OVR, STA, STS, and RS are highly correlated fruit characteristics with fruit size. Moreover, FLT and SMT fruit characteristics showed a lower positive correlation (r= 0.71) with fruit size. CYG, CG, and CMR were positively correlated with each other. However, there was a moderately negative correlation (r= -0.62) between CY and CR. Furthermore, there was a negative correlation (r= - 0.43) between CY and CDO as well (Figure 13). The correlations between related traits are consistent as expected in the phenotypic data. For example, characteristics related to fruit size were correlated with each other. Likewise, a correlation was observed between TA, AC, and pH, which are responsible for fruit acidity, as expected.

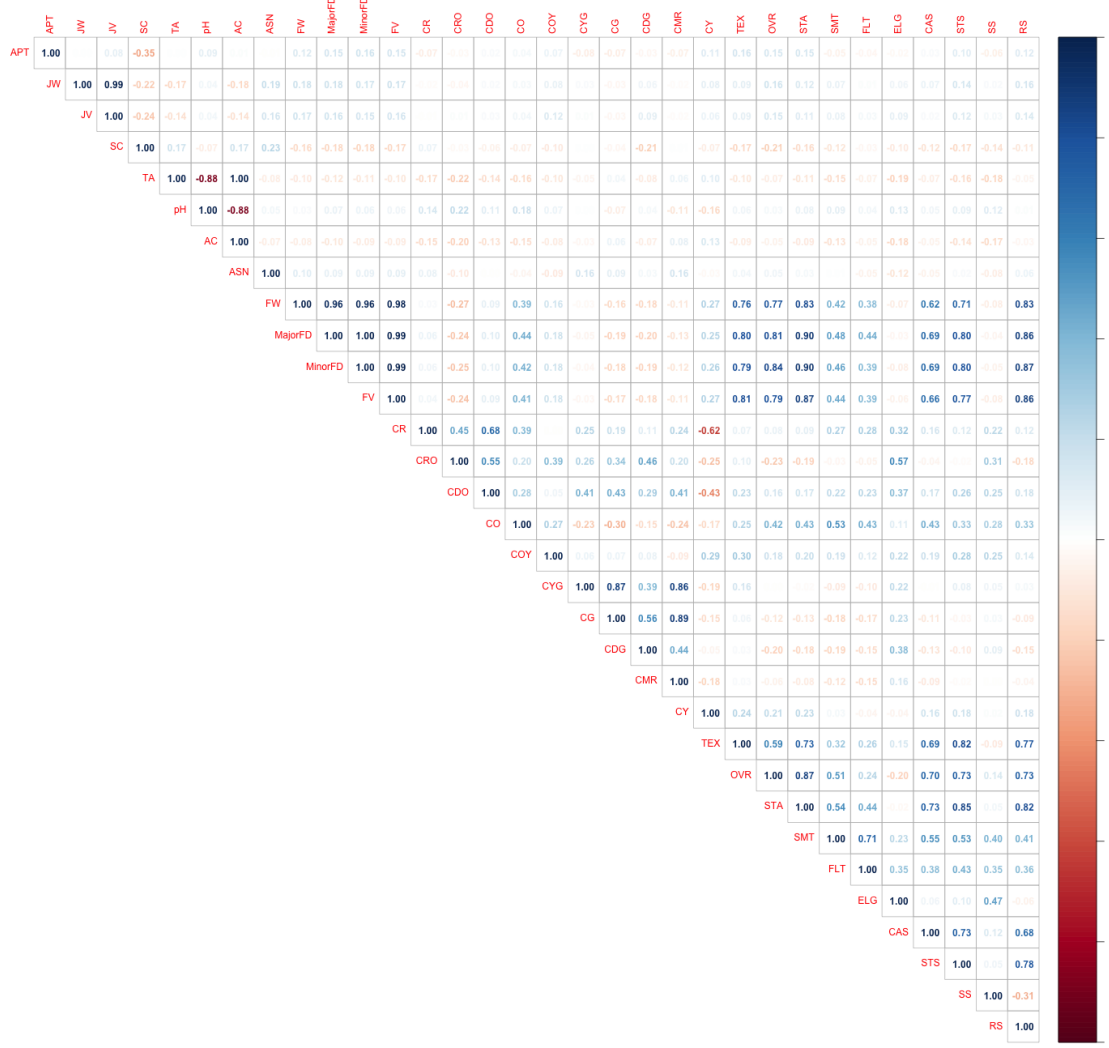


Figure 13. Spearman correlations between the fruit quality traits and fruit characteristics

SUMMARY OF EXTERNAL FRUIT COLOR VARIATION

A Principal component analysis (PCA) on the color trait values was performed for all samples (Figure 14, Figure A4). According to the PCA plots, most of the phenotypic variation in the population was explained by the first two PCs. The first two principal components captured 61% (PC1) and 22% (PC2) of the phenotypic variance.

The first component (PC1) separated individuals within the population based on their values for the CY and CO color traits. The second component (PC2) separated individuals within the population based on their values for the CMR and CG color traits. In addition, PC2 separated the parents (Figure 14), which are known to differ in the level of external red fruit color. The same PC plot, but variations in specific colors are highlighted with the yellow-purple shading. In other words, individuals of the population were separated with CY and CMR colors. Phenotypic variation in color was summarized using PC1 and PC2 values and these values were used in all downstream analyses.

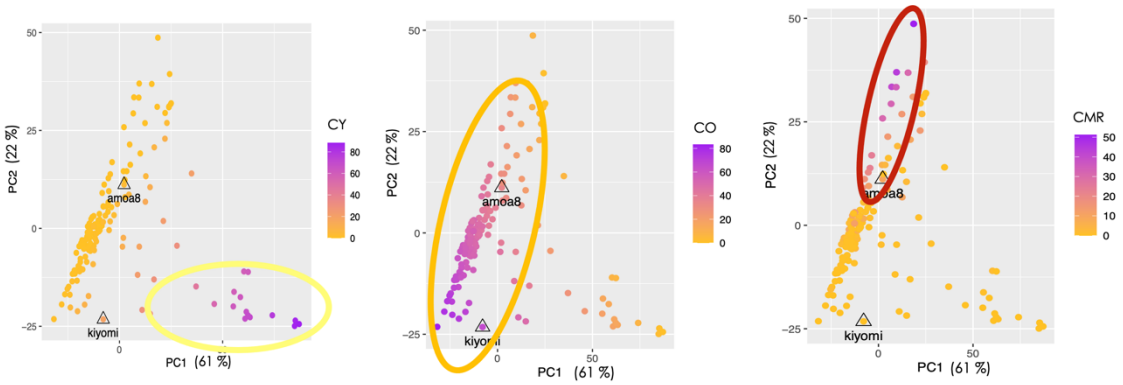


Figure 14. PCA analysis on color traits

THE EFFECT of YEAR-to YEAR VARIATION on TRAIT VALUES

Fruits were collected from field-grown trees that were subject to local weather conditions. The aim is to understand how robust traits measurements were across years and focused on two traits - SC and MajorFD.

To measure the stability of genetic effects across years, according to the measurements for 2020, twenty individuals were selected to represent the phenotypic extremes for two traits – SC and MajorFD. For each trait, 10 individuals with the highest trait values and 10 individuals with the lowest trait value were identified. SC and MajorFD were re-measured in fruit collected in 2021 for these 40 individuals. The measurements for each trait showed consistency between two years for each group, and hence genetic effects across years are stable (Figure 15). However, differences between the high and low groups were smaller in year 2 and during the year 1 when the groups were identified. This is consistent with an environmental contribution to variation in these traits.

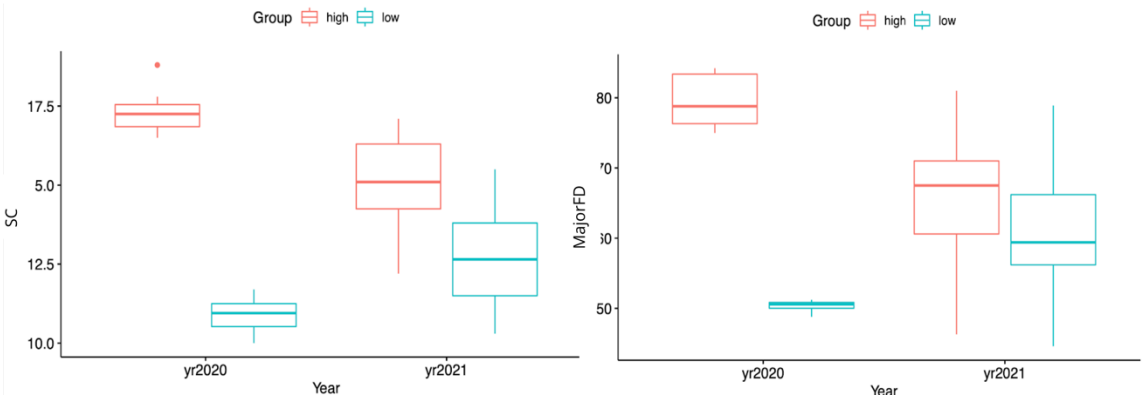


Figure 15. Selected the 20 individuals with the highest and lowest measurements for each of SC and MajorFD in 2020 and 2021

MARKER SEGREGATION and POLYMORPHISM

50064 SNP markers were genotyped, and 13418 SNP markers segregate between two parents. A total of 7303 markers are informative for mapping QTL derived from Kiyomi (homozygous in Amoa-8, heterozygous in Kiyomi). 4491 markers are informative for mapping QTL derived from Amoa 8 (i.e. homozygous in Kiyomi and heterozygous in Amoa 8). Of the 13418 SNP markers, 2510 markers heterozygous in Kiyomi and 1628 markers heterozygous in Amoa 8 had missing calls in one or more progeny (Figure 16). After missing data were removed, 5740 SNP markers (Figure 17 (a)) remained for Kiyomi and 3540 SNP markers (Figure 17 (b)) for Amoa 8. The number of individuals genotyped for each marker in both parents was 96 (Figure 17 (c) and (d)). The number of markers per chromosome is shown in Table 10. To construct the genetic maps with R/qtl, a backcross (BC) strategy was used. Thus, the expected segregation of the markers rate was 1:1. In the population, the individuals had genotype frequencies that are in approximately similar proportions in Kiyomi and Amoa 8, respectively AA was 51.5 %, 47.7 % and AB was 48.5% and 52.3%. As shown in Figure 18, the frequencies of heterozygous genotypes and homozygous genotypes met the expected genotype frequency of the population.

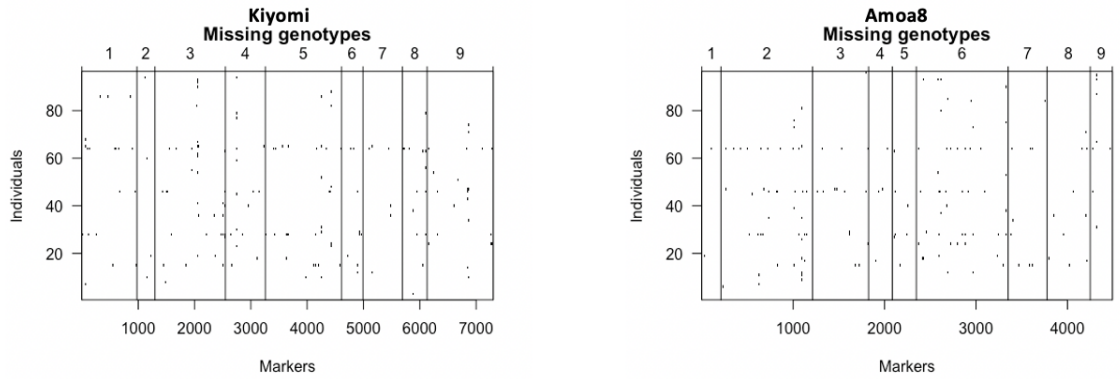


Figure 16. The plots of missing genotype data (Kiyomi and Amoa 8). Black pixels indicate missing genotypes.

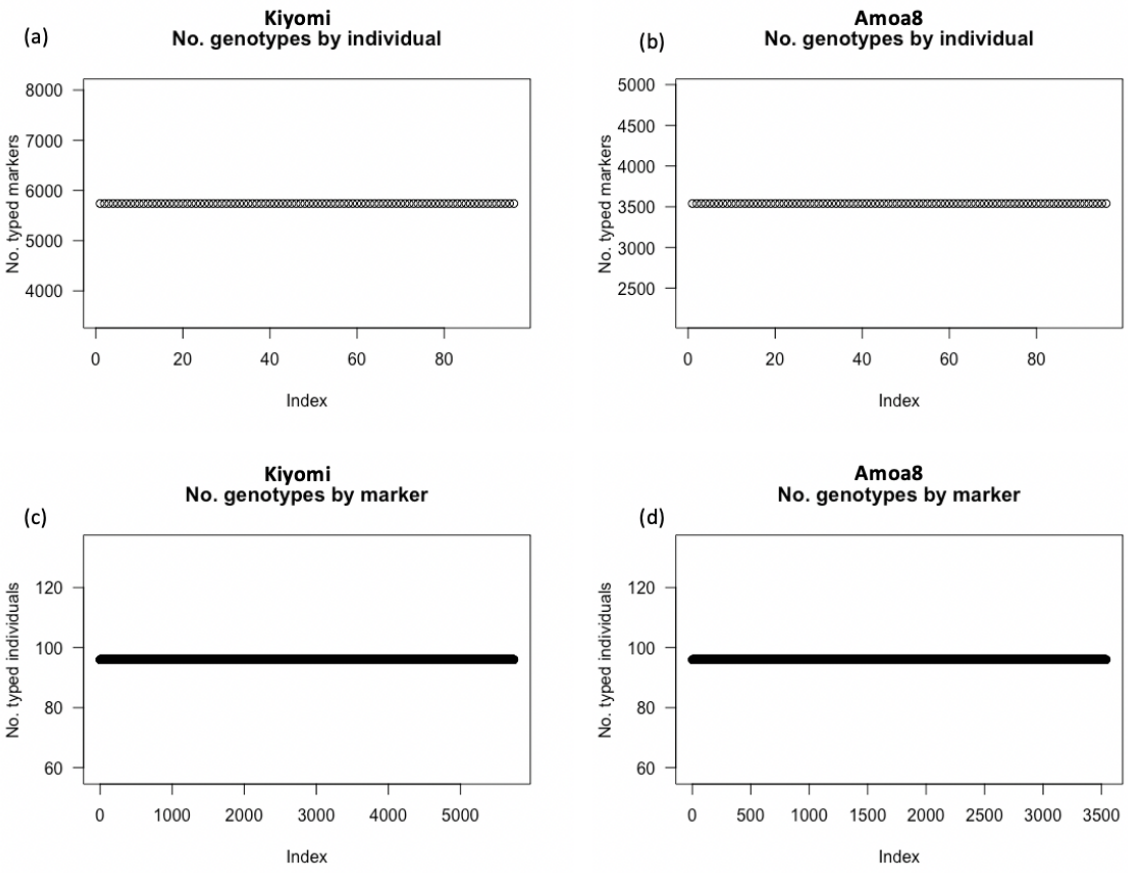


Figure 17. The plot of the number of genotyped individuals for each marker in Kiyomi (a) and Amoa 8 (b). The plot of the number of genotyped markers for each individual in Kiyomi (c) and Amoa 8 (d).

Table 10. The number of markers per chromosome

Genotypes	Kiyomi	Amoa 8
[0/0]	2164	3503
[1/1]	2327	3800
[0/1]	7303	4491
After markers with missing data removed	**5740**	**3540**
Chromosome 1	739	168
Chromosome 2	261	806
Chromosome 3	969	468
Chromosome 4	538	200
Chromosome 5	1056	205
Chromosome 6	288	797
Chromosome 7	566	336
Chromosome 8	345	377
Chromosome 9	978	183

[0/0] and [1/1] represented homozygous genotypes (AA), [0/1] represented heterozygous genotype (AB)

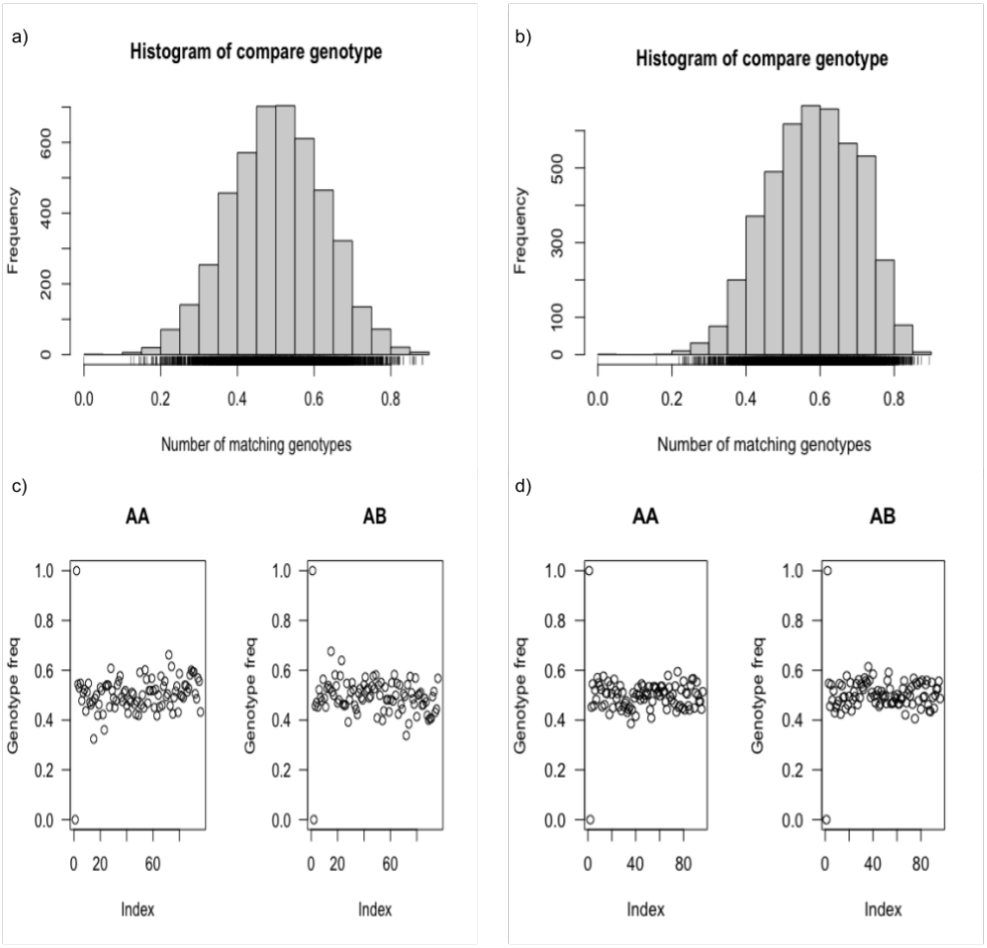


Figure 18. Histogram of the proportion of markers for which pairs of individuals have matching genotypes in Kiyomi (a) and Amoa 8 (b), Genotype frequencies by individual in Kiyomi (c) and Amoa 8 (d)

The recommended quality control procedures to filter markers prior to QTL mapping were followed according to two references (Broman and Sen (2009), Broman (2010). These procedures included removing distorted markers, identifying markers incorrectly placed, and removing duplicated and switched markers. After completing these procedures, the recombination fraction was estimated and a LOD score for the test of r = 0.5 for each pair of markers was calculated, and then crossing over numbers were counted. According to the plot of estimated pairwise recombination fractions, there were

not any problematic markers. It was most probably that markers were placed in their accurate position on chromosomes (Figure 19 (a) and (b)). There were no estimated recombination fractions that were r> 0.5, and no large recombination fractions with large LOD scores (Figure 19 (c) and (d)). There was a distribution between 0-30 crossing overs among the 96 individuals (Figure 19 (e) and (f)). Although there were some departures from 1:1 segregation on chromosomes 1 and 9, the estimated genetic maps were good to start constructing QTL mapping (Figure 20 (a) and (b)).

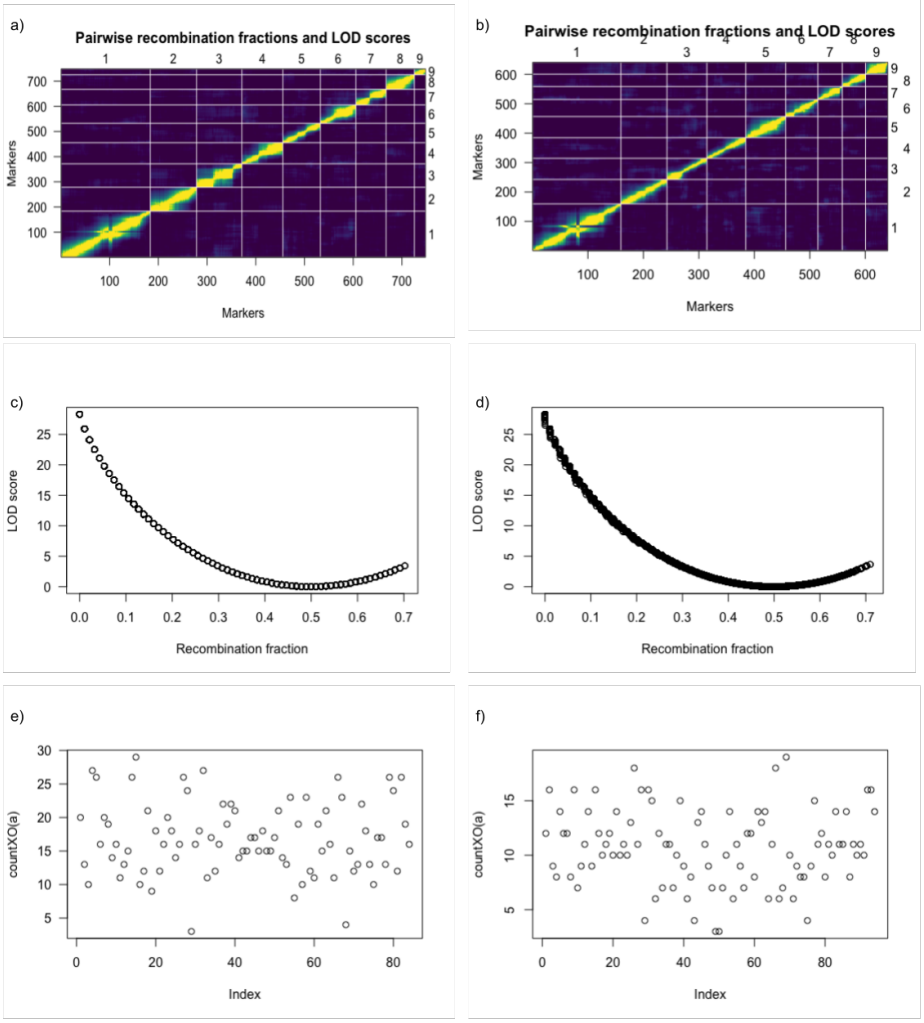


Figure 19. Estimated pairwise recombination fractions in Kiyomi (a) and Amoa 8 (b), LOD scores in Kiyomi (c) and Amoa 8 (d), and the number of crossing overs in Kiyomi (e) and Amoa 8 (f) for all pairs of markers. Yellow parts of the plot mean pairs of markers that appear to be linked (low rˆ or high LOD). Blue signifies no linked pairs (high rˆ or low LOD) (Broman and Sen 2009).

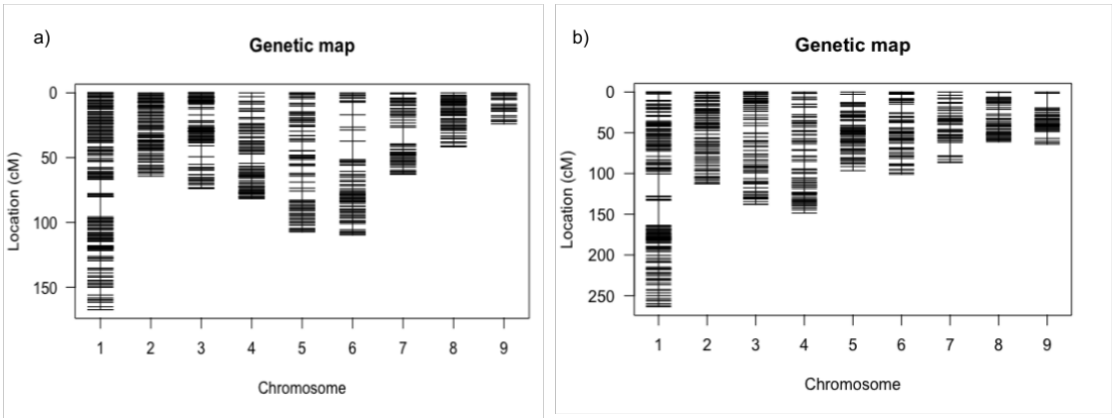


Figure 20: Plot of the final estimated genetic maps in Kiyomi (a) and Amoa 8 (b)

QTL MAPPING

The genetic maps were constructed by representing all nine chromosomes. There were 9 Linkage groups (LGs) and they corresponded to Clementine genome scaffolds. For Kiyomi map consisted of the final 750 SNPs, and the map for Amoa 8 consisted of the final 642 SNPs on nine LGs. The LGs that correspond to Clementine Scaffold are shown in Table 11. 8 QTLs were identified for the 32 fruit quality traits: 1 QTL was associated with APT, 1 QTL was associated with pH, 2 QTLs were associated with ASN, 2 QTLs were associated with TA, and 2 QTLs were associated with AC. 3 QTLs were identified in the female parent (Kiyomi) on LG 2. The QTL for ASN was positioned not only LG 2 but also LG 1 (Figure 21). A QTL was detected on LG 5 in Kiyomi for FC (Figure 22). However, any QTLs could not be identified in Amoa 8 for FC and 4 4 QTLs were identified in the male parent on chromosome 5 (Amoa8) (Figure 23).

Table 11. The location of the LGs according to the Clementine Scaffold

Kiyomi	**CHROMOSOME**	1	2	3	4	5	6	7	8	9
	LG	1	2	4	5	6	3	7	8	9
Amoa 8	**CHROMOSOME**	1,2	3	4	5	6	7	8	9	
	LG	1	3	4	2	8	5	9	7	

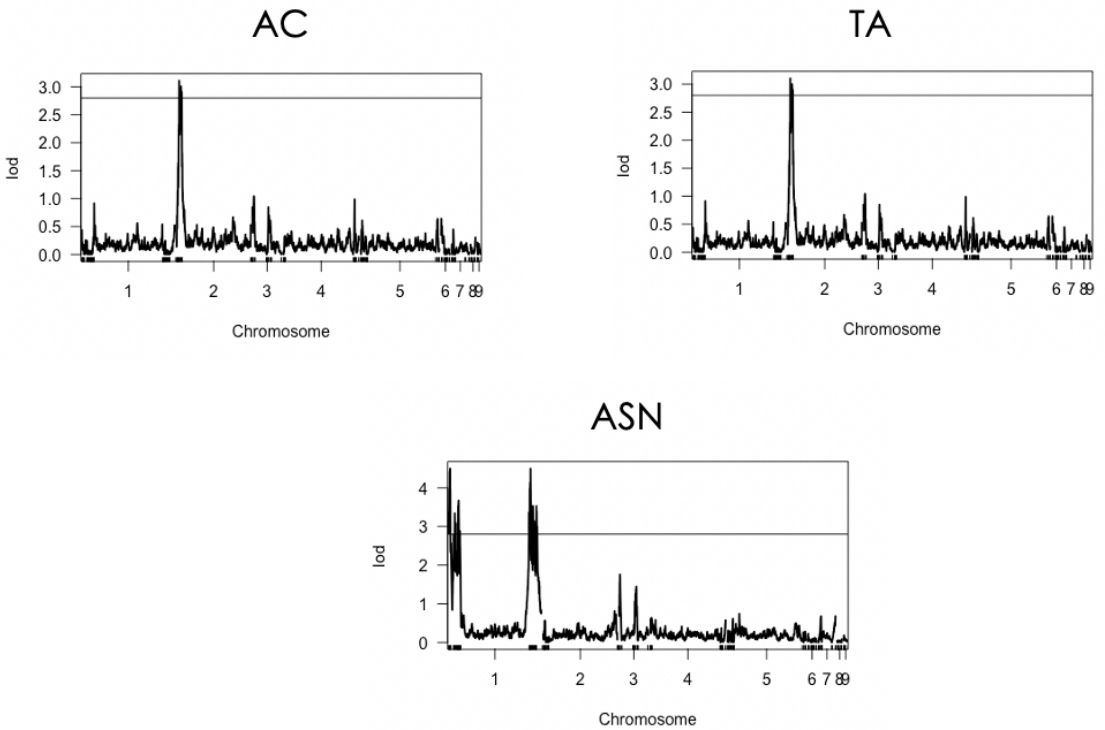


Figure 21. The identified QTLS in Kiyomi (LOD thresholds (1000 permutations) 5% 2.81, 10% 2.47)

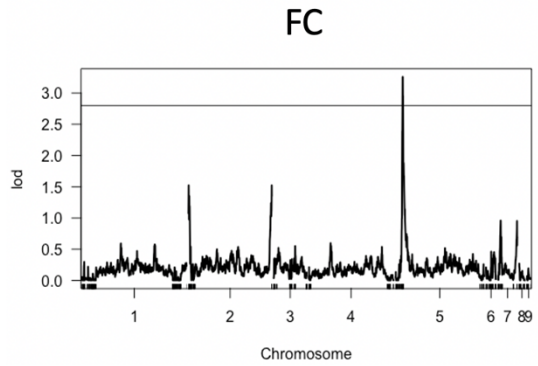


Figure 22. The identified QTL for FC in Kiyomi (LOD thresholds (1000 permutations) 5% 2.81, 10% 2.47)

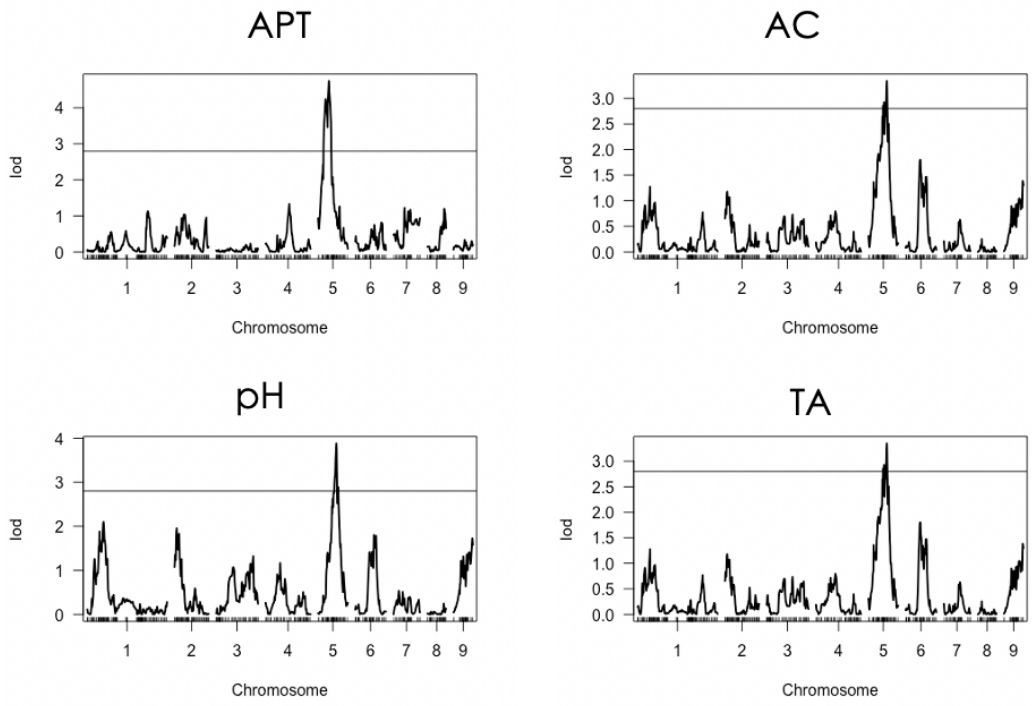
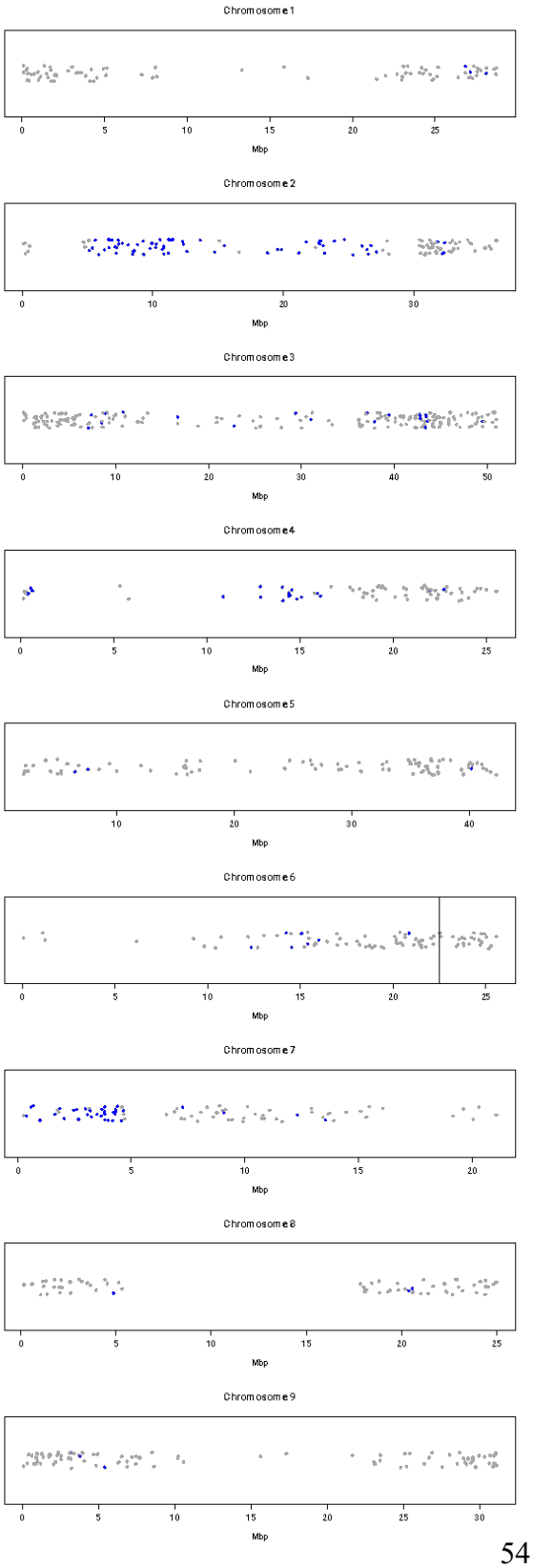


Figure 23. The identified QTLs in Amoa 8. (LOD thresholds (1000 permutations) 5% 2.90, 10% 2.58)

Amoa 8 is a tangor with intense red internal color. On this parent, there was not any QTLS for color traits. The switched and distorted markers were removed at the

beginning of the QTL analysis. The location of final markers for Amoa-8 across each chromosome was indicated in Figure 24. RUBY was marked with a horizontal line on chromosome 6. There were nearby markers so it is not yet clear why we did not detect QTL associated with this locus known to control anthocyanin accumulation in Citrus.


Chromosome 1
0
5
10
15
20
25
Mbp
Chromosome 2
0
10
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30
Mbp
Chromosome 3
0
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20
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50
Mbp
Chromosome 4
0
5
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15
20
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Mbp
Chromosome 5
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Chromosome 6
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Chromosome 7
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15
20
Mbp
Chromosome 8
0
5
10
15
20
25
Mbp
Chromosome 9
0
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20
25
30
Mbp

Figure 24. The location of final markers for Amoa-8 across each chromosome. Distorted markers are shown in blue. RUBY was marked with a horizontal line on chromosome 6.

DISCUSSION

Our study identified AC, TA, and ASN QTLs from parent Kiyomi and APT, TA, AC, and pH QTLs from parent Amoa 8. Each group of focal traits and how detected QTL relate to prior research on each trait will be briefly discussed.

Fruit Size

In previous QTL studies associated with fruit size, 8 QTLs for fruit weight and 3 QTLs for fruit diameter were determined by Yu et.al (2016). These QTLs on the scaffolds 4, 5, and 8 of the Clementine reference genome explained phenotypic variance from 15.03 % to 24.6%. The highest LOD score of the QTL for fruit weight on scaffold 8 of the Clementine reference genome was 2.91 in this study. Imani et. al (2017) reported that 5 QTLs for fruit weight on the scaffolds 3, 4, and 7 of the Clementine reference genome range phenotypic variance from 14.9 % to 26.5%. Among these QTLs, the LOD score was 3.68. Fruit diameter is a trait responsible for fruit size as well. Yu et. al (2016) identified 3 QTLs associated with fruit diameters 4, 5, and 9. The highest LOD score was 2.9 among 3 QTLs. In our study, although we study 32 fruit quality traits, we did not identify any QTLs for fruit size. In addition, Imai et. al 2017 claimed that FW4.2 (Yu et. al 2016) and FWq3 (Imai et. al 2017) might correspond on scaffold 4 of the Clementine genome due to their same position. However, we did not detect any QTLs for fruit size and in the same position.

Fruit flavor

The sugar content and acidity of the fruit are responsible for fruit quality. Yu et. al 2016 reported that the QTLs identified for SSC (^{O}Brix) on the scaffolds 2, 3, 4, and 8 of the Clementine genome. Among these QTLs, the highest LOD score was 2.92. A QTL was detected for SSC on scaffold 5 of the Clementine genome, with the highest LOD score of 2.80 by Imani et. al 2017. For TA, Yu et. al (2016) identified QTLs on scaffolds 7, 8, and 9 on the Clementine reference genome. The highest LOD score was reported as 2.99. In our study, although we could not identify any QTLs for sugar content, we detected QTLs associated with TA, acid content, and pH. We have seen that in the distributions of TA, AC, and pH, the offspring are highly transgressive with many individuals beyond the range of the parents. These features are highly correlated with each other (Figure 11, Figure B2). As a result of mapping for these features, QTLs in Kiyomi were on scaffold 2, while for Amoa 8 they were in scaffold 5 of the Clementine reference genome. The QTLs detected in our study did not correspond to the QTLs detected in previous reference studies on TA. In our study about fruit acidity, 3 more QTLs were detected. These were associated with AC and pH. The QTL was positioned on scaffold 5 for pH in Amoa 8 and the QTLs were located on scaffolds 2 in Kiyomi and 5 of the Clementine genome for AC in Amoa 8.

Seedlessness

4 QTLs related to the number of seeds were determined. Three of them were detected on the parent in Fortune Cl7a, Cl7c, and Cl10. In the study, it was stated that most of the offspring had a high seed number. In our study, the number of seeds in the population varied from 0 to 32.58, but it was 9.18 on average (Figure B2). Yu et. al (2016) identified 2 QTLs for SN on scaffolds 3 and 9 of the Clementine genome with LOD scores of 2.94 and 2.89, respectively. The QTLs detected in our study did not include any for SN.

Fruit Color

Sugiyama et. al (2011) detected 3 QTLs for all total carotenoids. They were located on scaffolds 6 and 7 of the Clementine reference genome. Asins et. al (2015) measured flavedo color and fruit juice color for fruit color properties by using a chromatic circle. They identified 4 QTLs for fruit color in the parent Fortune and 4 QTLs for fruit color and 3 QTLs for juice color in the parent Chandler. Yu et. al (2016) measured flavedo color and fruit juice color for fruit color properties (FCA, FCAB, FCB, FCL, JCA, JCAB, JCB, JCL). Flavedo color and juice color were measured with a colorimeter. 28 QTLs were identified for fruit color. The QTLs for fruit color with the highest significant levels were all found in the Murcott parent. Among these 8 QTLs, all except for 1 QTL (FCA8) are on scaffold 4 of the Clementine genome. Fruit color is one of the most important fruit quality characteristics. In our study, 10 fruit skin colors were measured. PCA analysis of these was performed and instead of 10 color features, PC1

and PC2 were used for color traits for QTL mapping. None of the QTL mapping results gave a significant peak for fruit color traits. Therefore, we could not detect any QTL for fruit skin color in our study. For the flesh color, one QTL was determined for FC, which was measured by associating it with the red color of the fruit on Kiyomi but not on Amoa 8.

Peel or Rind Thickness

For peel or rind thickness, 4 QTLs were identified by Asins et. al (2015). These QTLs were on Cl7a, Cl3, Cl7a, and Cl4b in the parent Fortune. We identified a QTL for average peel thickness (APT) on the scaffold 5 of the Clementine genome in only Amoa 8.

Juiciness

Juiciness was determined by 2 QTLs by Asins et. al (2015) on CL3 and CL4b. Yu et. al(2016) detected only one QTL (JP 7.2) for juice volume on the scaffold 7 of the Clementine genome. We did not identify any QTL for JV.

Surprisingly, there was no correlation between JW, JV and FW, FV in our study. This made us think about the possibility that the sampled fruits could be drier.

DISCOVERED QTL and THEIR RELATIONSHIP to PRIOR RESEARCH

The QTLs were discovered only for a small subset of traits in our study. It can be thought that one of the main reasons is related to the effect of population size on QTL

detection. In previous studies, 201 full sibs (Asins et. al 2015), 116 F1 hybrids (Yu et. al 2016), and 110 F1 hybrids were used as population. In our study, 100 individuals were used but 96 individuals were evaluated. A typical QTL population consists of 100 to 300 individuals. Large effected QTLs in a smaller population can be identified but the QTLs of smaller effect can be identified in a larger population ("Population Sizes for QTL Studies").

We may not have detected QTL for color traits because of the way that color was determined. Each pixel was assigned one color. The color traits were detected by the software. There may have been an error in the separation between pixels by the software. For example, green (CG) and moro red (CMR) colors had highly positive correlations (Figure 13). This might explain this situation. In addition, the RUBY gene regulates anthocyanins accumulation in Citrus. Under cold conditions, this gene is upregulated, and the anthocyanins levels increase in fruits. The fruit color feature is highly dependent on the environmental conditions and changes accordingly. Thus, it makes it difficult to detect genetic control of the color traits. The reason for defining QTLs for Moro red and red features can be explained in this way. Although there were not a lot of distorted markers on Chromosome 6, a QTL colocalizing with RUBY was not identified on Amoa 8. Although Amoa 8 is a tangor with intense red internal color and there were not a lot of distorted markers on Chromosome 6 (Figure 24), RUBY did not underlie any color QTL. There could be several possible reasons. ex. RUBY may not be the only gene that controls anthocyanins accumulation in this population. We may not be able to detect RUBY has a QTL because there are no markers in linkage with RUBY. Another

possibility is that the sequence surrounding the RUBY mutation does not vary within the population, so it would not be detected as a QTL. Another reason could be related to fruit maturity. The fruits were harvested based on the maturity time of the parents and sent for analysis. Anthocyanin accumulation from offspring that do not have the same maturity period as their parents may not have been fully realized in hybrids. Color formation in immature fruits may not have occurred sufficiently. The Citrus fruit maturity can be calculated according to the Australian Citrus Standard (^{O}Brix-(%Acid x 4)) x 16.5) (California Department of Food and Agriculture, 2012). Since acidity and sugar rate determine fruit maturity, The high acid concentration in immature fruits may have prevented the coloration of fruits.

FUTURE WORK

For the next possible study, the population can be created with more individuals. The RUBY gene can be sequenced by Sanger sequencing in the remaining individuals from the same population. Fruit juice color with the help of HPLC, the amount of anthocyanin and carotenoids in the fruits can be determined for future genetic analysis of fruit color.

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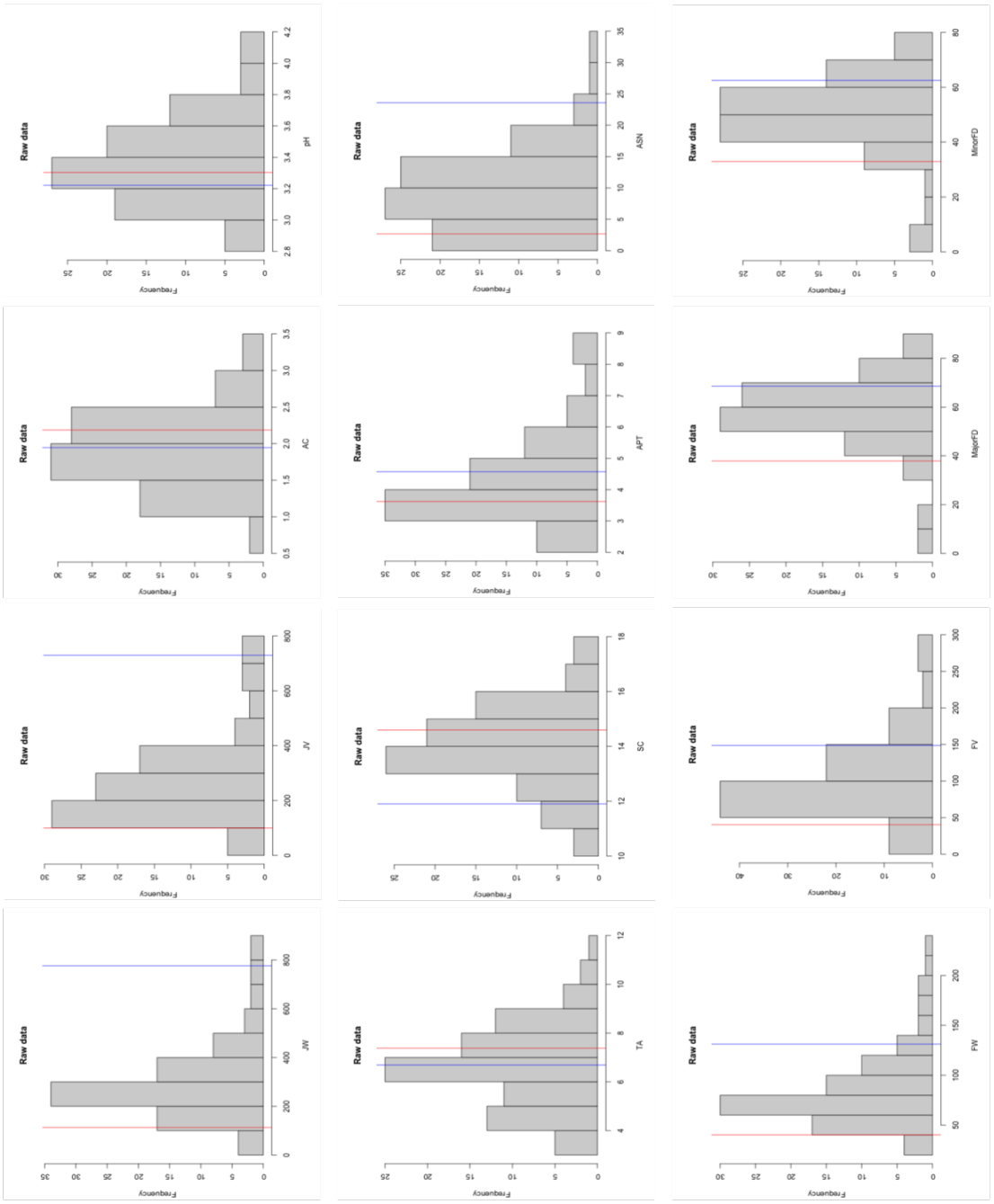

Figure A1. The Distributions of destructive and fruit size traits (Raw data). The parents Kiyomi and Amoa 8 were represented by the blue and red color lines, respectively

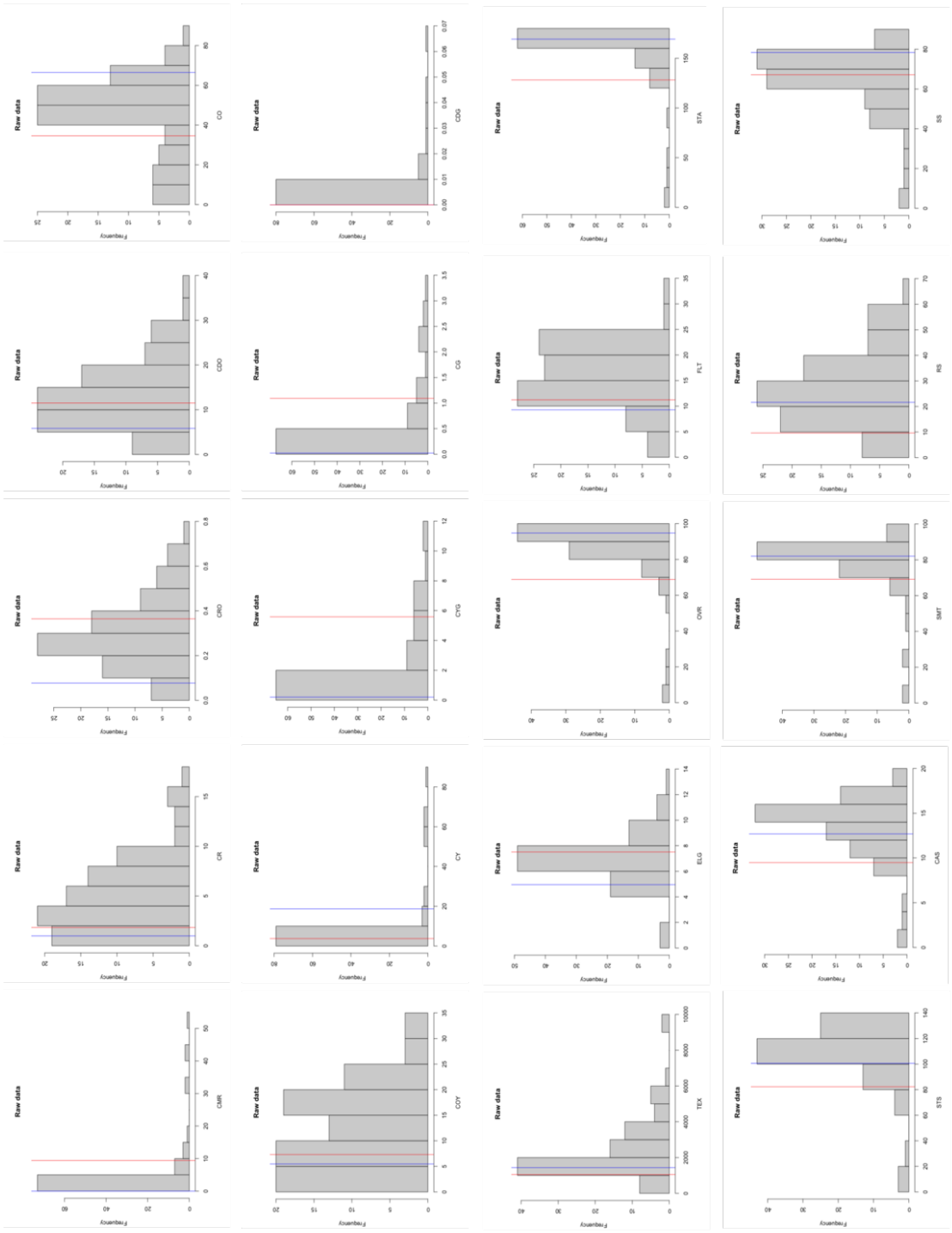

Figure A2. The Distributions of color (CMR, CR, CRO, CDO, CO, COY, CY, CYG, CG, CDG) traits and fruit characteristics (TEX, ELG, VOR, FLT, STA, STS, CAS, SMT, RS, and SS) (Raw data). The parents Kiyomi and Amoa 8 were represented by the blue and red color lines, respectively

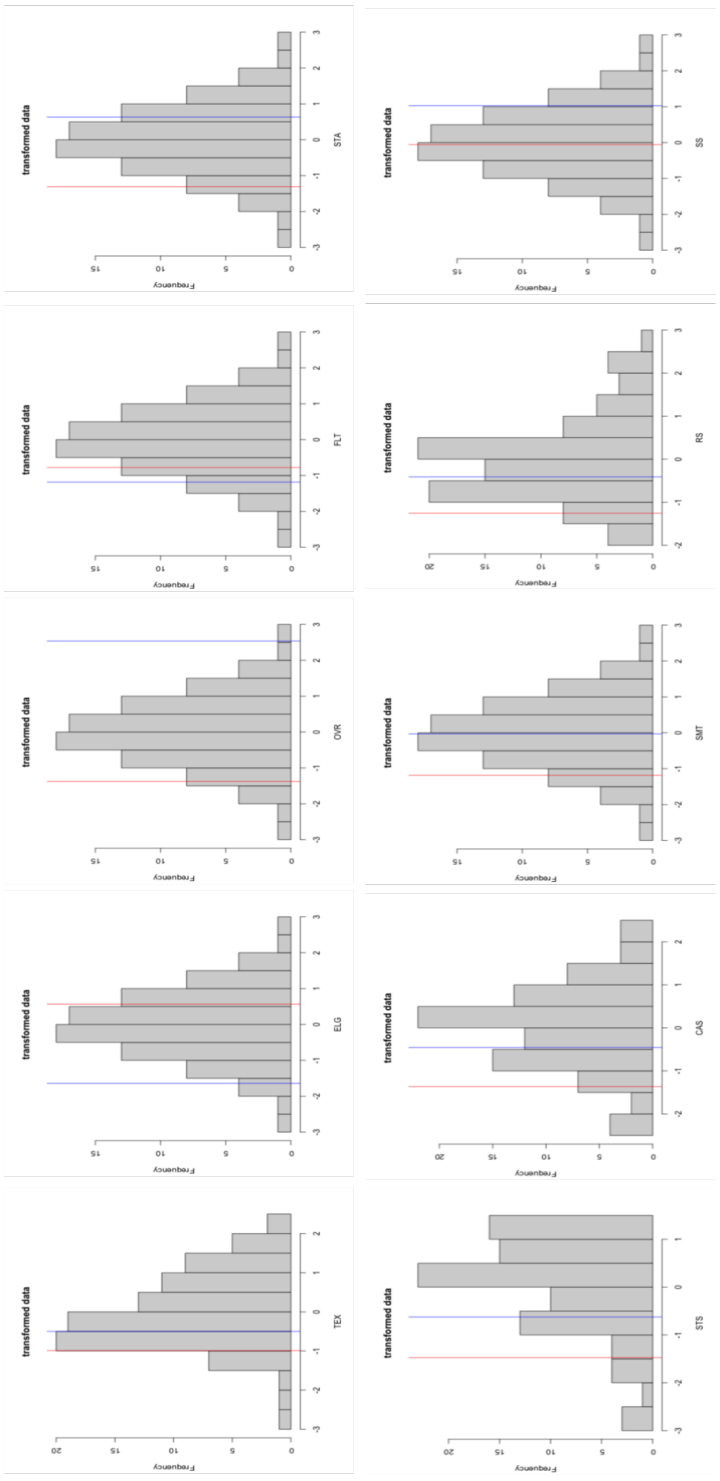

Figure A3. The distributions of TEX, ELG, VOR, FLT, STA, STS, CAS, SMT, RS, and SS (Transformed data). The parents Kiyomi and Amoa 8 were represented by the blue and red color lines,respectively

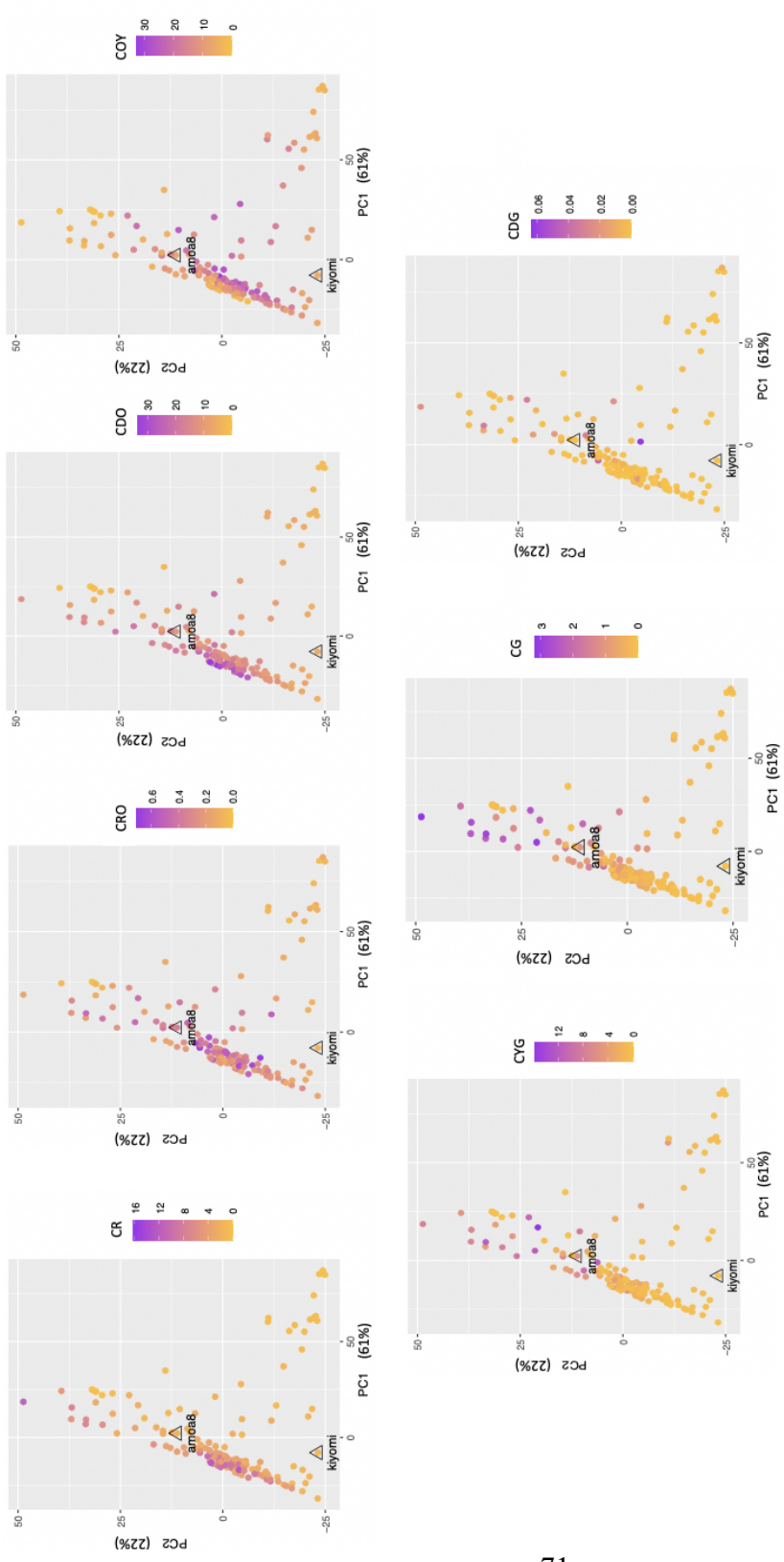
COY
CDO
CRO
CR
CDG
CG
CYG
PC2 (22%)
PC1 (61%)
amoa8
kiyomi

Figure A4. The principal component analyses on the color trait values were performed for all samples. Each plot highlights differences in single color values to determine which color traits are more closely associated with the PCs

Table B1. Summary of descriptive statistics for fruit quality traits and fruit characteristics evaluated

Phenotypes	n	mean	sd	median	min	max	range	skew	kurtosis	se
APT	157	4.47	1.46	4.12	1.99	9.48	7.49	1.29	1.86	0.12
JW	156	290.9	153.14	260.5	55	825	770	1.43	2.42	12.26
JV	149	273	143.2	240	80	770	690	1.56	2.64	11.73
SC	155	13.95	1.61	13.8	10	17.8	7.8	0.07	-0.16	0.13
TA	156	6.71	1.93	6.51	3	14.24	11.24	0.61	0.73	0.15
pH	157	3.4	0.36	3.36	2.9	6.46	3.56	3.99	31.08	0.03
AC	157	1.95	0.59	1.9	0.17	4.22	4.05	0.46	1.01	0.05
ASN	157	9.18	6.4	8.58	0	32.58	32.58	0.97	0.99	0.51
FW	154	83.11	36.51	74.41	20.63	226.14	205.51	1.21	1.86	2.94
MajorFD	154	55.44	16.28	57.91	1.78	84.21	82.44	-1.3	1.88	1.31
MinorFD	154	48.84	14.67	50.66	1.7	76.63	74.93	-1.1	1.58	1.18
FV	154	97.14	51.87	87.55	4.63	273.04	268.41	0.93	1.25	4.18
CR	154	4.34	3.42	3.53	0	16.19	16.19	1.17	1.21	0.28
CRO	154	0.28	0.16	0.26	0	0.73	0.73	0.58	-0.1	0.01
CDO	154	12.9	7.18	12.35	0	35.54	35.54	0.61	0.25	0.58
CO	154	42.67	19.55	47.74	0	83.23	83.23	-0.6	-0.49	1.58
COY	154	11.81	7.73	10.85	0	34.27	34.27	0.53	-0.52	0.62
CYG	154	2.02	2.71	0.78	0	15.97	15.97	2.35	6.17	0.22
CG	154	0.5	0.66	0.2	0	3.17	3.17	1.99	3.49	0.05
CDG	154	0	0.01	0	0	0.06	0.06	4.24	21.67	0
CMR	154	4.08	8.97	0.27	0	51.06	51.06	3.04	9.58	0.72
CY	154	8.36	19.66	0.49	0	88.22	88.22	2.76	6.59	1.58
TEX	154	2367	1651	1926.1	288.9	9883.5	9594.5	1.76	4.11	133
OVR	154	82.05	19.05	89.72	2.32	94.74	92.42	-2.6	6.75	1.53
STA	154	151.5	35.44	167.17	3.8	174.07	170.27	-2.6	6.55	2.86
SMT	154	75.92	18.32	82.01	2.22	94.22	92	-2.3	5.17	1.48
FLT	154	14.99	6.27	14.78	0.09	31.31	31.21	-0.1	-0.28	0.51
ELG	154	6.76	2.13	6.68	0.07	14.83	14.76	-0	2.29	0.17
CAS	154	13.19	3.65	14.09	0.51	19.8	19.29	-1.4	2.46	0.29
STS	154	103.5	27.35	112.29	2.32	129.69	127.37	-2.1	4	2.2
SS	154	63.33	16.31	67.4	2.38	92.04	89.66	-1.6	2.96	1.31
RS	154	26.46	13.69	26.13	0	69.76	69.76	0.5	0.18	1.1

Table B2. Best-Normalization methods evaluated for each phenotypic trait

Trait	Transformation	Non-Missing Obs
APT	Standardized Yeo-Johnson Transformation	157
JW	orderNorm Transformation	156
JV	Standardized asinh(x) Transformation	149
SC	Standardized asinh(x) Transformation	155
TA	Standardized Yeo-Johnson Transformation	156
pH	Standardized Box Cox Transformation	157
AC	center_scale(x) Transformation	157
ASN	orderNorm Transformation	157
FW	Standardized sqrt(x + a) Transformation	154
MajorFD	Standardized Box Cox Transformation	154
MinorFD	orderNorm Transformation	154
FV	orderNorm Transformation	154
CR	Standardized Yeo-Johnson Transformation	154
CRO	Standardized Yeo-Johnson TransformatioN	154
CDO	Standardized Yeo-Johnson Transformation	154
CO	orderNorm Transformation	154
COY	Standardized sqrt(x + a) Transformation	154
CYG	orderNorm Transformation	154
CG	Standardized Log_b(x + a) Transformation	154
CDG	orderNorm Transformation	154
CMR	orderNorm Transformation	154
CY	Standardized Log_b(x + a) Transformation	154
TEX	Standardized Box Cox Transformation	154
OVR	orderNorm Transformation	154
STA	orderNorm Transformation	154
SMT	orderNorm Transformation	154
FLT	Standardized Box Cox Transformation	154
ELG	orderNorm Transformation	154
CAS	Standardized Yeo-Johnson Transformation	154
STS	orderNorm Transformation	154
SS	orderNorm Transformation	154
RS	center_scale(x) Transformation	154