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Brief Report

Quantitative Trait Locus Analysis in Squash (*Cucurbita moschata*) Based on Simple Sequence Repeat Markers and Restriction Site-Associated DNA Sequencing Analysis

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Abstract: Squash (*Cucurbita moschata*) displays wide morphological and genetic variations; however, limited information is available regarding the genetic loci of squash that control its agronomic traits. To obtain basic genetic information for *C. moschata*, an F_2 population was prepared derived from a cross between the Vietnamese cultivar 'Bí Hồ Lô TN 6 (TN 6)' and the Japanese cultivar 'Shishigatani', and flowering and fruit traits were examined. Overall, the traits showed a continuous distribution in the F_2 population, suggesting that they were quantitative traits. A linkage map was constructed based on simple sequence repeat and restriction site-associated DNA (RAD) markers to detect quantitative trait loci (QTLs). Twelve QTLs for flowering and fruit traits, as well as one phenotypic trait locus, were successfully localized on the map. The present QTLs explained the phenotypic variations at a moderate to relatively high level (16.0%–47.3%). RAD markers linked to the QTLs were converted to codominant cleaved amplified polymorphic sequence (CAPS) and derived CAPS markers for the easy detection of alleles. The information reported here provides useful information for understanding the genetics of *Cucurbita* and other cucurbit species, and for the selection of individuals with ideal traits during the breeding of *Cucurbita* vegetables.

Keywords: flower and fruit traits; QTL; RAD-seq; SSR

1. Introduction

Squash, pumpkin, and gourd species (*Cucurbita* spp.) are vegetables of the genus *Cucurbita* belonging to the family Cucurbitaceae. This family also includes vegetables of economic importance

such as cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.), and watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai). Cucumber is thought to have originated in India and is enjoyed either fresh or pickled. The origin of melon is unclear, whereas watermelon originated in Africa. Melon and watermelon are mainly eaten for their sweet flesh. These three cucurbits have low nutritional value compared with other vegetables, but provide ranges of sweetness, texture, and color [1]. The genus *Cucurbita* contains five domesticated species, three of which are major, *C. maxima* Duch., *C. moschata* Duch., and *C. pepo* L., with two minor species (*C. argyrosperma* K. Koch and *C. ficifolia* Bouché). *Cucurbita* is native to the New World, first domesticated there approximately 10,000 years ago; currently, it is cultivated for its fruits and seeds worldwide [1]. Fruits of this species display wide variations in morphology with regard to size, shape, and color. The flesh and seeds contain nutritionally valuable compounds, sugars, carotenes, and oils, the contents of which vary according to the varieties and growth conditions. Despite such merits, genetic and genomic research on *Cucurbita* remain limited compared with cucumber, melon, and watermelon. Although most of its traits are still being investigated at the level of conventional genetics [2], some trait loci have been localized to linkage maps [1,3]. Moreover, whole-genome data have recently been made available for the three major *Cucurbita* species [4–6].

Among the three major *Cucurbita* species, relatively extensive studies have been carried out in *C. pepo* because this species exhibits the widest morphological and genetic variation as well as the highest economic importance. *C. moschata*, the next most variable species in this genus, is distributed to tropical, subtropical, and temperate regions, and has adapted to a humid tropical climate. Genetic loci for fruit traits have been mapped in *C. moschata* [7–10], but only limited information is available. Since the introduction of *C. moschata* to Japan in the 16th century, multiple local cultivars and landraces have been differentiated there, rendering it one of the centers of *C. moschata* diversity [11]. 'Shishigatani', a Japanese local *C. moschata* cultivar, is an heirloom vegetable in Kyoto Prefecture [12]. This cultivar bears a large, dumbbell-shaped fruit that is believed to have been bred from a flattened-fruit variety. The dumbbell-shaped fruit of 'Shishigatani' has been reported to be recessive to flattened fruit [13,14]. Moreover, the phytohormone auxin affected its fruit development [14]. However, the detailed genetic mechanisms underlying its fruit shape remain unknown. Many agronomic traits, including fruit size and shape, are quantitative and can be controlled by multiple loci, called quantitative trait loci (QTLs) [1].

Molecular markers are powerful tools for classification, genetic mapping and breeding. Several molecular markers have been developed and utilized in *Cucurbita* [1]. Of these, simple sequence repeats (SSRs) or microsatellites, which are repeated sequences of 1–6 nucleotide motifs, are used for classification and linkage analysis because of their advantages, such as simple detection of DNA variation (polymorphism), relatively high reproductivity and polymorphism, and codominant inheritance [15]. Recently, a next-generation sequencing approach has also been adopted for the genetic analysis of *Cucurbita*. Restriction site-associated DNA sequencing (RAD-seq) is one such method [16], which involves massive parallel sequencing of DNA fragments at particular positions after restriction digestion and adapter ligation, to obtain many nucleotide polymorphisms around the restriction sites with a relatively low cost.

To obtain basic genetic information for *C. moschata* especially for 'Shishigatani', several crosses among squash cultivars were tested, and it was found that the Vietnamese cultivar 'Bí Hồ Lô TN 6 (TN 6)' was an ideal source for genetic analyses because of its compact fruit size with a sweet flesh. The external color of the mature fruits of 'TN 6' and 'Shishigatani' is buff, but their flesh colors are intense orange and yellow, respectively (Figure 1A,B and Figure S1A,B). A linkage map was constructed in an F_2 population derived from a cross between 'TN 6' and 'Shishigatani' based on SSR and RAD markers to survey QTLs for agronomic traits. This resulted in the successful detection of several QTLs that control flower and fruit traits. The aim of this study was to construct a linkage map and to identify QTLs for flower and fruit traits in squash, for understanding the genetics of *Cucurbita* and other cucurbit species, and for the selection of *Cucurbita* vegetables.

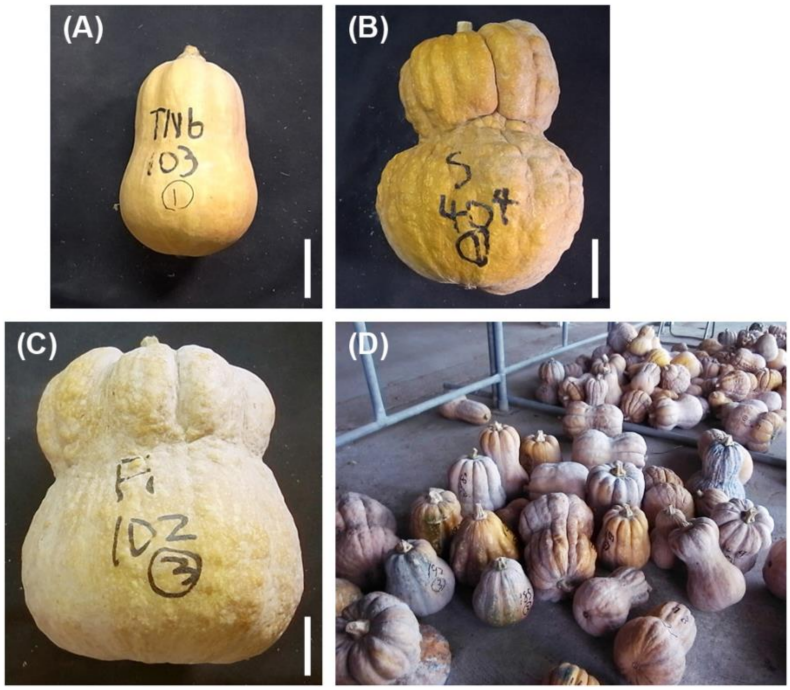

Figure 1. Morphologies of mature fruits at harvest (ca. three months after transplanting) for the parental lines and F_2 plants used in this study. (**A**) 'Bí Hồ Lô TN 6 (TN 6)'; (**B**) 'Shishigatani'-K; (**C**) F_1; and (**D**) F_2 plants. A single individual is shown as a representative of the parental and F_1 lines, respectively. Scale bar, 5 cm.

2. Materials and Methods

2.1. Plant Materials and Field Tests

An F_1 plant was derived from a cross between the Vietnamese ('TN 6') and Japanese cultivars of squash (*C. moschata*) ('Shishigatani', line name: 'Shishigatani'-K, maintained at the Agriculture and Forestry Technology Department, Kyoto Prefectural Agriculture, Forestry and Fisheries Technology Center, Kameoka, Japan), as maternal and paternal parents, respectively. 'TN 6' is a commercial, non-hybrid cultivar (Trang Nông Seeds Co., Ltd., Ho Chi Minh, Vietnam) that produces a small, pyriform fruit with blotchy, cream-colored spots on the pericarp at the immature stage (Figure 1A and Figure S1C, left). 'Shishigatani' is a Japanese landrace and a heirloom vegetable in Kyoto Prefecture that bears a large, dumbbell-shaped fruit with heavy warts and pleats on the surface (Figure 1B). Because 'TN 6' is an open pollinated cultivar, some loci were heterozygous. However, it was possible to predict its genotype from the residual genotype of 'Shishigatani'-K, as 'Shishigatani'-K is highly homozygous because of inbreeding (data not shown). An F_2 population was generated via the selfing of the F_1 plant, resulting in 369 F_2 seeds from the single F_1 fruit. Seeds of 88 F_2 plants, F_1, and the parental lines (three or four seeds for each parental line) were sown in pots with a diameter of 9 cm in April 2016 and cultivated for three weeks in a greenhouse. They were transplanted at the end of April, 2016, to an open field of the University Farm, Faculty of Life and Environmental Sciences, Kyoto Prefectural University (Soraku-gun, Kyoto, Japan, 34.7733° N, 135.7600° E, altitude 102 m). Fertilizer (N, 4.7 g; P_2O_5, 4.7 g; and K_2O, 4.2 g/m^2) and compost (2 kg/m^2) were applied prior to transplanting according to the vegetable cultivation standard of the Department of Agriculture, Forestry and Fisheries, Kyoto Prefecture. Plants were grown under natural conditions in plots with a row length of 100 cm and 55 cm between each plant. Two vines (one main and one secondary vine) were left on each plant and any other vines were removed. One to seven fruits per plant (average 2.4) were produced under natural pollination.

2.2. Scoring of Traits

Flowering traits were scored with regard to the flowering time and days after transplanting to the opening of the first male and female flowers (Table S1). At the immature fruit stage (Figure S1C), the presence/absence of cream-colored spots on the pericarp was scored using binary data (presence = 1, absence = 0) for incorporation into a linkage map as a morphological trait marker (Mottled skin). After three months of cultivation in the field, fruits were harvested and stored at room temperature until the scoring of fruit traits (Table S1). The following fruit traits were examined: fresh weight; height; circumferences of the upper, middle, and lower parts (Figure S2, Up, Mid, and Low, respectively); and degree of ribs and wart (fruit surface texture) on the pericarp. Ratios of circumferences on upper/middle and lower/middle parts were calculated (Up/Mid and Low/Mid, respectively). Flesh color was measured with regard to three parameters, L^* (lightness or darkness), a^* (red/green coordinate), and b^* (yellow/blue coordinate) using a colorimeter (CR-400; Konica Minolta Japan, Inc., Tokyo, Japan). These parameters were scored randomly at three points on the flesh surface for each fruit. Subsequently, C^* (chroma: color saturation or intensity) and hue (hue angle: relative amounts of redness and yellowness) were calculated according to the previous report [17]. Brix was measured using a sugar refractometer PEN-J (Atago, Co., Ltd., Tokyo, Japan). Trait data were subject to statistical tests using BellCurve for Excel 2.20 (Social Survey Research Information, Tokyo, Japan).

2.3. Genotyping Analyses, Construction of a Linkage Map, and QTL Analysis

DNA was extracted from a fresh leaf of the F_2 population and parental lines according to the previous report [18]. The genotypes of each line were analyzed using *C. moschata* and *C. pepo* SSR markers [19] (Table S2). SSR fragments were amplified by polymerase chain reaction with fluorescence-labeled primers (Sigma-Aldrich, St Louis, MO, USA) and analyzed on a CEQ8000 DNA sequencer (Sciex, Vaughan, Canada), as reported previously [18]. RAD-seq analysis was performed on 88 F_2 individuals and their two parental lines, as per the previous report [18]. Briefly, a DNA library was made from DNAs digested with *Bgl*II and *Eco*RI, followed by adaptor ligation, purification, and sequencing of 50 bp single-end reads using a HiSeq 2000 (Illumina, San Diego, CA, USA). The RAD-seq reads were subjected to mapping to the squash genome (*C. moschata* 'Rifu') [4] as a reference data, according to the previous report [18]. The sequence data have been deposited in the DDBJ Sequence Read Archive database under accession no. DRA010639. Any marker locus containing 30 or more missing data out of the 88 F_2 individuals or significantly deviated ($P \leq 0.00001$) from the expected segregation ratio (1:2:1 and 3:1 for codominant and dominant markers, respectively) in the F_2 population was removed from subsequent analysis. No imputation of missing genotypes from RAD-seq analysis was performed because no apparent improvement was observed in the construction of a linkage map (data not shown). A linkage map was constructed from the genotype data of SSR and RAD markers plus one morphological trait marker (Mottled skin), as reported previously [20]. The QTL analysis was performed using MapQTL 6 [21] under the multiple-QTL model option, which is equivalent to composite interval mapping, as reported previously [20]. The logarithm of odds (LOD) thresholds for each QTL ($\alpha = 0.05$) were estimated by 1000 permutations. The QTL-linked RAD markers were converted to cleaved amplified polymorphic sequence (CAPS) and derived CAPS (dCAPS) markers based on the homology to the squash genome sequence [4].

3. Results

3.1. Traits of the F_2 Population Derived from a Cross between 'TN 6' and 'Shishigatani'-K

To investigate the flower and fruit traits of squash, an F_2 population was generated by crossing two *C. moschata* cultivars, 'TN 6' and 'Shishigatani'-K, to obtain sufficient phenotypic and genetic variations for linkage mapping and QTL analysis. The traits of 88 F_2 plants, F_1, and its parental lines measured in this study included flowering time, texture of fruit surface, fresh weight and size of fruits, flesh color, and Brix (see Table S1 for details). The surface (rib and wartiness) of fruit from F_1 plants resembled

those of 'Shishigatani'-K (Figure 1B,C and Figure S3C,D). The constriction in the middle part of F_1 fruits was relatively more similar to that of 'Shishigatani'-K vs. 'TN 6' (Figure 1A–C and Figure S3K). Variations in fruit traits were observed in the F_2 fruits (Figure 1D). Most of the traits showed continuous distributions (Figure S3), suggesting that they were quantitative traits. Among them, five (Up–Mid, Up–Up/Mid, Low–Low/Mid, a^*–C^*, and b^*–C^*) and two (Fw–H and Mid–Low) pairs of traits showed strongly and moderately positive correlations, respectively (Table 1, red and orange boxes, respectively). Two (Mid–Up/Mid and a^*–b^*) and one (Mid–Low/Mid) pairs of traits showed strongly and moderately negative correlations, respectively (Table 1, blue and light-blue boxes, respectively).

3.2. Construction of a Linkage Map Based on SSR and RAD Markers Using the F_2 Population

DNA polymorphisms in the F_2 populations were detected based on the SSR markers and RAD-seq analysis. Thirteen SSR makers out of the 126 tested showed polymorphisms and were localized on the linkage map. A total of 160,191,488 reads were obtained in the F_2 population by the RAD-seq analysis, 131,881,811 of which were used for mapping to the reference genome. A total of 143,309 polymorphic loci and 179,173 SNPs were detected (1628.5 polymorphic loci and 2036.1 SNPs per F_2 individual on average). After removal of any marker locus containing missing data (≥30) or significantly deviated segregation ratio, 794 RAD markers were finally used for the construction of a linkage map. The resulting linkage map was composed of 36 linkage groups (LGs) containing 443 loci (13 SSRs, 20 CAPS or dCAPS markers, 409 RAD markers, and one morphological trait marker (Mottled skin)) (Figure S4). The total map length was 2026.7 cM, with an average marker interval of 4.6 cM. The RAD markers used in this study basically formed LGs according to the pseudochromosomes of the *C. moschata* genome [4]. SSR markers were mapped to the LGs as reported previously [4,8] (Table S2), with two exceptions (CMTm232b and CMTp216b). Although the number of LGs was not converged to that of the expected haploid chromosomes (n = 20), it was assumed that the present map covered the *C. moschata* genome sufficiently for QTL analysis, based on the comparison of map length between the present (2026.7 cM) and previous studies (1268.4–3087.0 cM) [7–10].

3.3. QTL Analysis of Traits in the F_2 Population, and Development of CAPS and dCAPS Markers for the Genotyping of Alleles

On the present map, a single QTL was identified for each of the traits, L^*, b^*, C^*, Low/Mid, Low, Wart, Brix, F flwr, Up/Mid, Up, Fw, and H (Table 2 and Figure S4, colored box). Of these, each of three pairs of QTL (L^*–b^*–C^* and Up–Fw–H) were located in the close vicinity of LGs 2a and 20, respectively. QTLs for Low/Mid, Low, and Wart were detected in the same LG (4a), but they were separated. The level of explanation of the phenotypic variations by the detected QTLs was moderate to relatively high (16.0%–47.3%). QTLs for L^*, Low/Mid, Low, Wart, Up/Mid, Up, Fw, and H yielded negative additive effects (Table 2), suggesting that they were derived from the 'Shishigatani'-K allele. The RAD markers near the present QTLs could be converted to codominant CAPS and dCAPS markers (Table 3), resulting in easier detection of alleles compared with the original RAD markers.

Table 1. Correlation of traits in the F_2 population.

	M Flwr	**F Flwr**	**Rib**	**Wart**	**Fw**	**H**	**Up**	**Mid**	**Low**	**Up/Mid**	**Low/Mid**	**L***	**a***	**b***	**C***	**hue**	**Brix**
M flwr																	
F flwr	0.3323																
Rib	0.1034	0.0770															
Wart	−0.2359	0.1257	0.4140														
Fw	−0.0915	0.1763	0.0019	−0.2632													
H	−0.0053	−0.2309	−0.0660	0.2628	0.7171												
Up	−0.0155	−0.0519	−0.1242	0.0858	0.2428	−0.0490											
Mid	0.0075	−0.1373	0.1242	−0.0309	0.0895	−0.2405	0.7576										
Low	0.1033	0.1466	−0.0042	0.1254	0.2132	0.0515	−0.0614	0.5475									
Up/Mid	0.0503	0.0410	0.1766	−0.0589	−0.0400	0.0047	0.9544	−0.7712	−0.0226								
Low/Mid	0.0170	−0.2510	−0.0609	0.0439	0.1732	−0.2347	−0.0599	−0.5611	0.8608	0.0327							
L*	0.3315	−0.2338	−0.1551	0.3412	0.0543	−0.0343	−0.0582	0.0766	−0.1288	0.0400	−0.0373						
a*	−0.1102	−0.3193	0.0714	0.1075	0.3050	−0.3831	−0.0423	−0.1577	0.1216	0.0249	−0.1610	0.0439					
b*	−0.1113	−0.2916	0.0406	0.0169	0.1500	−0.3085	0.0840	−0.2851	0.2434	−0.0620	−0.2400	0.0754	−0.8784				
C*	0.1181	0.2947	−0.0450	−0.0225	−0.1602	0.3149	−0.0732	0.2760	−0.2386	0.0532	0.2350	−0.0823	0.8965	0.9991			
hue	0.0004	−0.0651	0.0817	0.1816	0.3644	−0.2298	−0.2209	0.1893	−0.2132	0.1429	0.1287	0.0081	−0.4389	0.0332	0.0049		
Brix	−0.0559	0.1992	0.0706	0.0336	−0.0493	0.0962	−0.1888	0.2024	−0.0226	0.1759	0.0204	−0.0328	0.0853	0.1961	−0.1878	−0.1839	

The abbreviations of traits refer to those in Table S1. Correlation coefficient: 1.00 ≥ > 0.75 ≥ > 0.50 ≥ > 0.25 ≥ ≥ −0.25 > ≥ −0.50 > ≥ −0.75 > ≥ −1.00.

Table 2. Quantitative trait loci (QTLs) for flowering and fruit traits detected in the F_2 plants derived from a cross between 'TN 6' and 'Shishigatani'-K.

Trait [1]	LG [2]	Map Position in cM (peak) [3]	LOD [4]	α (%) [5]	Additive Effect [4]	Dominance Effect [4]
L*	2b	5.7–13.6 (7.6)	13.2	47.3	−3.75	−1.23
b*	2b	5.7–13.6 (7.6)	7.8	34.4	3.39	3.06
C*	2b	5.7–11.6 (6.7)	6.2	28.8	3.39	3.02
Low/Mid	4a	4.0–7.9 (6.0)	4.9	22.9	−0.13	−0.01
Low	4a	14.8–21.8 (17.8)	4.4	20.6	−3.24	−4.10
Wart	4a	46.2–46.5 (46.2)	6.8	31.0	−0.44	−0.34
Brix	6b	0.0–3.9 (3.9)	4.8	23.1	1.57	−0.26
F flwr	7	74.0–80.6 (78.6)	4.8	23.0	3.14	2.71
Up/Mid	8b	2.2–2.4 (2.4)	4.8	17.4	−0.08	−0.01
Up	20	31.9–35.9 (33.9)	4.2	16.0	−3.70	6.28
Fw	20	34.9–45.0 (43.0)	5.5	25.0	−0.39	0.35
H	20	35.9–44.0 (39.9)	4.4	16.8	−1.42	1.34

[1] The abbreviations of traits refer to those in Table S1. [2] Linkage group (LG), on which QTLs were localized in the present map. [3] Region above the logarithm of odds (logarithm of odds (LOD)) threshold, in which the peak position is indicated in parenthesis. [4] Scores at the LOD peaks. [5] Phenotypic variation explained at the LOD peak.

Table 3. List of codominant cleaved amplified polymorphic sequence (CAPS) and derived CAPS (dCAPS) markers developed and modified in this study.

Marker Name [1]	Primer Sequence (5′-to-3′) [2]	LG [3]	Trait Near the Marker	Restriction Enzyme	Expected Size (bp) [4]
CAPS and dCAPS markers converted from restriction site-associated DNA (RAD) markers in this study					
020880Chr02-CAPS	Forward: CCCGTCTTAGTAGTAGTAACAGAAA Reverse: CTCAAGTATTGATTTCGAATAGGTCC	2b	L*, b*, C*	*Tsp*509I	'TN 6': 11 + 19 + 37 + 150 'Shishigatani'-K: 11 + 19 + 37 + 47 + 103
021081Chr02-dCAPS	Forward: CAGGTAATAGCCATTGATGAATTTC Reverse: GAAAGCAGCAGCATCTTTCTGgAT	2b	L*, b*, C*	*Eco*RV	'TN 6': 24 + 108 'Shishigatani'-K: 132
023266Chr03-CAPS	Forward: CAGAAGTAGATGAAAAGTAGAACGACG Reverse: GTTCGAATTCAACCCTGGTTCTTTTG	3	Mottled skin	*Mbo*I	'TN 6': 235 'Shishigatani'-K: 47 + 50 + 138
025799Chr04-dCAPS	Forward: CCTCTCCAACTAATGTGAGATagTAC Reverse: GTGTAATAAAGCAGGTGCAGTAACAT	4a	Low/Mid	*Sca*I	'TN 6': 24 + 126 'Shishigatani'-K: 150
026418Chr04-dCAPS	Forward: GTTAAACTCAAAGGATAAGTATGGGT Reverse: AGAAAGTCTACTTGTAGCTATTTTCcC	4a	Low	*Sma*I	'TN 6': 27 + 103 'Shishigatani'-K: 130
027253Chr04-dCAPS	Forward: CAATGATATCTTAGATCTTCATTTTGCAtT Reverse: CCTGTCAACATTTAAATTCACAGATAT	4a	Low	*Mse*I	'TN 6': 14 + 28 + 29 + 82 'Shishigatani'-K: 14 + 28 + 111
027595Chr04-CAPS	Forward: GCATTACTTGAATAAAATCAATGTTAGAC Reverse: CATGAACCTATACTTCAAGACAAGTTAC	4a	Low	*Nla*III	'TN 6': 39 + 95 'Shishigatani'-K: 134
027863Chr04-dCAPS	Forward: GGAGATCCGCTGAAATCGcC Reverse: CGTCGACGATCTTTGGAGAATTC	4a	Low	*Nae*I	'TN 6': 19 + 129 'Shishigatani'-K: 148
033391Chr04-dCAPS	Forward: ATTGAACAAGCCTCATCAATCGTTtcTA Reverse: GCAAATGCATTTTGGAATTTCGTATTAAG	4a	Wart	*Xba*I	'TN 6': 152 'Shishigatani'-K: 25 + 127
034024Chr04-CAPS	Forward: TGGTTTAGGATCAAGCCACTAGA Reverse: AACACCACCCTTAAATTTGAAGCAC	4a	Wart	*Rsa*I	'TN 6': 160 'Shishigatani'-K: 65 + 95

Table 3. *Cont.*

Marker Name [1]	Primer Sequence (5′-to-3′) [2]	LG [3]	Trait Near the Marker	Restriction Enzyme	Expected Size (bp) [4]
041970Chr06 -CAPS	Forward: TAGAATAAGGAGATTCGAAATCCAG Reverse: GAAGGAATTCCCATGGGAATCAATG	6b	Brix	*Ssp*I	'TN 6': 142 'Shishigatani'-K: 47 + 95
045451Chr07 -CAPS	Forward: CATTGGGAATTCAGATTTAGATCTG Reverse: GAATTCATCGCTAAGCTTCTCGA	7	F flwr	*Taq*I	'TN 6': 22 + 183 'Shishigatani'-K: 22 + 63 + 120
045491Chr07 -CAPS	Forward: CAATCGAATTTTGCAGGCAAAACAAGT Reverse: GTAGTTCAGGTTGCTCTAATCAATTTC	7	F flwr	*Xba*I	'TN 6': 86 + 188 'Shishigatani'-K: 274
045492Chr07 -CAPS	Forward: GATAGGAAACGATATCAGTATTGAGATC Reverse: GAGTTTATGTTCAAGTCGGTGATATTAG	7	F flwr	*Mbo*I	'TN 6': 24 + 52 + 130 'Shishigatani'-K: 24 + 182
047940Chr08 -dCAPS	Forward: GAACATCTCATACTGGTTGAAGAG Reverse: TTTGGAAATGTTTTCCCACCTTTATCtA	8b	Up/Mid	*Xba*I	'TN 6': 138 'Shishigatani'-K: 29 + 109
048046Chr08 -dCAPS	Forward: AGATCTTCATTGAATTATTACAATGGTTgA Reverse: GCCATTCTATTTTTAATCTGTTGATTTGA	8b	Up/Mid	*Hin*fI	'TN 6': 166 'Shishigatani'-K: 29 + 137
048147Chr08 -dCAPS	Forward: TCACCGCTGGTAGATATTGTCA Reverse: TGAAGACGTGCATGTAATCCCaATT	8b	Up/Mid	*Mun*I	'TN 6': 174 'Shishigatani'-K: 25 + 149
103408Chr20 -CAPS	Forward: TTCAAGCCCATTGCTAGCAGATA Reverse: TGGGAACGTCATTTGTATTTATACTG	20	Up, Fw, H	*Xba*I	'TN 6': 49 + 101 'Shishigatani'-K: 150
Simple sequence repeat (SSR) marker developed in this study					
CmoChr03 -SSR1	Forward: GGAATACTGTAAGAAGATATGCCGA Reverse: CCCATTAAGAATACAATAGAACCTTG	3	Mottled skin	–	'TN 6': ca. 110 'Shishigatani'-K: ca. 120
dCAPS markers (Zhou et al. *2018) modified in this study*					
R1_47757 -dCAPS	Forward: AAATAAGGTTGTCGAATTATCcTGcA Reverse: TCCTGAAGTGGACAACGAACTA	3	Mottled skin	*Pst*I	'TN 6': 26 + 168 'Shishigatani'-K: 194
R2_63809 -dCAPS	Forward: TTCCAACAATTTCCCTCTACTGC Reverse: TTGCTATTTTCTTGCATTCGATATCtcT	3	Mottled skin	*Xba*I	'TN 6': 223 'Shishigatani'-K: 30 + 193

[1] CAPS = cleaved amplified polymorphic sequence; dCAPS = derived CAPS. [2] Small case letters represent sequence deviations from the original sequences for introduction of the restriction sites. [3] Linkage group (LG), on which QTLs were localized in the present map. [4] Sizes of fragments are shown for each allele of the two parental lines ('TN 6' and 'Shishigatani'-K).

4. Discussion

In this study, a genetic analysis of agronomic traits was performed by measuring several flowering and fruit traits to map their underlying genetic loci. The population size of F_2 plants used in this study (88) was slightly small compared with that used in the other crops, although a sufficient amount of F_2 seeds (369) was obtained for genetic analysis. This was because *Cucurbita* samples required a large area for their cultivation. The large size of the plants such as *Cucurbita* (typically 0.2–0.6 m tall and >10 m in length) makes them ill-suited to genetics studies [22]. A greater population size (≥100) could improve the resolution of the present linkage map. Nevertheless, 12 QTLs and one phenotypic trait locus (Mottled skin) were successfully localized on the present map (Table 2 and Figure S4). Of these, QTLs for Wart (degrees of wartiness on the fruit pericarp) and F flwr (days after transplanting to the opening of the first female flower) were newly mapped in *C. moschata*. The appearance of F_1 fruits was similar to that of 'Shishigatani'-K fruits in this study (Figure 1C), suggesting that the fruit surface traits (rib and wartiness) of 'Shishigatani' are dominant in relation to those of the 'TN 6', which bears the

smooth fruits. This is in good agreement with reports that demonstrated that the presence of wartiness was a dominant trait in *Cucurbita* species [22]. A direct comparison of the genomic positions between the present and previous genetic loci in *C. moschata* [7–10] based on the SSR and RAD markers was impossible for most of the loci because no specific markers and/or no common traits were examined. No QTL for flesh color and carotenoids overlapped between the previous [9,10] and the present studies. A similar result was found in traits pertaining to Brix and sugar content. Such observations may suggest the cultivar-specific control of the traits examined here.

A Mottled skin locus for cream-colored spots on the fruit pericarp at the immature stage was inherited in a single dominant manner and was mapped on LG 3 in this study (Figure S4). A similar single dominant gene has been reported in *C. moschata* [9,23], in which the genomic location of Mldg (Mottled light and dark green immature fruit color) [23] is unknown because of the lack of map information. The other locus, pc (pericarp color), has been mapped on pseudochromosome 3 (corresponding to LG 3 in this study) [9] and its markers have been developed [24]. Of these, markers R1_47757 and R2_63809 were mapped near the Mottled-skin locus in the present map (Figure S4). The close location between the pc markers and the Mottled-skin locus strongly suggests that both loci are identical or are in the same vicinity. Fine-mapping and map-based cloning approaches would be able to isolate its candidate gene sequence(s).

One of the objectives of this study was to examine the inheritance of the "dumbbell" fruit shape, which is a unique characteristic of the Japanese heirloom cultivar 'Shishigatani'. To attain this objective, the F_2 population derived from a cross between cultivars with pyriform ('TN 6') and dumbbell-shaped fruits ('Shishigatani') was used. In addition to the reasons described above, the choice of using cultivars with somewhat similar fruit shapes was because a complex variation in fruit shape had been found in the F_2 population derived from a cross between the parents with very different fruit shapes (i.e., 'Shishigatani' × a flattened-fruit cultivar without any neck), which hampered the evaluation of fruit shape in the F_2 offspring [13], as well as the identification of loci associated with this trait. Similar difficulties were observed in the head formation trait of Chinese cabbage and the problem has been solved using two heading types of Chinese cabbage [20,25]. The dumbbell-shaped fruit of 'Shishigatani' was surely recessive to flattened fruit, as reported previously [13,14], because an F_1 hybrid between 'Shishigatani' and a flattened fruit cultivar ('Hoko aokawakuri') bore fruit without a neck (data not shown). In contrast, the appearance of fruits from F_1 individuals ('TN 6'× 'Shishigatani'-K) was relatively similar to that of 'Shishigatani'-K (Figure 1C), suggesting that the constriction in the middle part of fruits is completely or partially dominant to the pyriform shape in this squash cross. Using the present F_2 population, QTLs for the fruit shape regarding the constriction in the middle (Up/Mid and Low/Mid) and other fruit size-related traits (Up, Low, and H) were detectable on LGs 4a, 8b, and 20 in this study (Figure S4). Although a fruit size-related QTL (fruit diameter: qfd8-a) has been mapped on pseudochromosome 8 [9], the correspondence of Up/Mid and qfd8-a is currently unclear because of the lack of detailed marker information [9]. Regarding fruit shape, the involvement of the *Ovate* family protein gene has been reported in *C. pepo* and bottle gourd [26–28]. *Ovate* is a gene that controls elongated fruit growth in tomato [29]. Any evidence of the involvement of the *Ovate* homolog currently has not been obtained.

Only a single QTL for each of the 12 traits was detectable in this study. This might mean the involvement of QTLs with a small effect, in addition to the single large-effect QTL, which could have hampered the stable detection of QTLs. Assuming that the present QTLs explained the phenotypic variations at a moderate to relatively high level, the QTLs detected here seem to be relatively stable. Further study may confirm the stability and reproductivity of such QTLs. The CAPS and dCAPS markers linked to the QTLs could be applied to the selection of individuals in breeding programs of squash. The findings reported here provide useful information for understanding the genetics of *Cucurbita* and other cucurbit species, and for the selection of individuals with ideal traits during the breeding of *Cucurbita* vegetables.

5. Conclusions

Flowering and fruit traits were measured in a *C. moschata* F_2 population derived from a cross between two squash cultivars (Vietnamese cultivar 'TN 6' and Japanese cultivar 'Shishigatani'-K). A linkage map was constructed based on SSR and RAD markers in the F_2 population, on which 12 QTLs for flowering and fruit traits as well as one phenotypic trait locus were localized. Of these, QTLs for Wart (fruit wartiness) and F flwr (days of the first female flower) were newly mapped in *C. moschata*. QTLs for fruit shape regarding the constriction in their middle (Up/Mid and Low/Mid) and other fruit size-related traits (Up, Low, and H) were also identified. RAD markers linked to the QTLs were converted to CAPS and dCAPS that are useful for selection of individuals during the breeding programs of *Cucurbita* vegetables.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-7524/6/4/71/s1, Figure S1: Trait for flesh color and cream-colored spots on the pericarp, Figure S2: Schematic representation of measurement of fruit-size related traits, Figure S3: Frequency distributions of the traits scored for the F_2 population of 'TN 6' × 'Shishigatani'-K, Figure S4: A linkage map of the F_2 population ('TN 6' × 'Shishigatani'-K). Table S1: List of traits analyzed, and the phenotypic values of the F_2 population and the parental lines, Table S2: List of *Cucurbita* simple sequence repeat (SSR) markers localized on the linkage map in this study.

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