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Construction of dense genetic linkage maps of apple cultivars Kaşel-41 and Williams' Pride by simple sequence repeat markers

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Abstract: Almost all commercially grown apple cultivars in Turkey are exotic, with local varieties serving as important genetic resources for breeding and marker development purposes. Among the latter is Amasya, which has a pleasing taste and flavor, but is unsuitable for commercial use because of its small fruit size and alternate-bearing characteristics. Genetic maps of apple cultivars are important resources to facilitate marker-assisted selection. Because of its good eating quality, Amasya is a good genetic resource for marker development to enhance fruit quality, especially flavor. In this study, we constructed a simple sequence repeat (SSR) marker-based genetic map of the Amasya apple cultivar using 96 F1 progenies generated from a cross between Kaşel-41 (a selection from Amasya) and Williams' Pride cultivars. Of 187 bacterial artificial chromosome SSRs, 150 expressed sequence tag SSRs, and of 362 genomic SSR primer pairs, 400 markers were mapped, with parental and consensus maps constructed independently. A reference map was also constructed using 207 common SSR markers, thereby producing the most dense SSR-based reference map currently reported for apple. The reference genetic map was 1351 cM long, with a mean marker density of 6.5 cM. The consensus map obtained by mapping 396 markers had a length of 1476.4 cM and a 3.82-cM mean marker density. We also identified the positions of 57 SSR loci previously unmapped in apple. As a result, once additional markers have been incorporated into the maps to increase saturation, the evaluation of phenotypic traits in the mapping population should facilitate linkage between molecular markers and fruit quality traits. Additionally, the constructed apple reference genetic map, with 207 SSR markers, should also assist the construction of new genetic maps and aid integration of various apple linkage maps.

Key words: Apple, *Malus*, simple sequence repeat, microsatellite, genetic mapping

1. Introduction

The cultivated apple (*Malus* × *domestica* Borkh.) is one of the most economically important temperate fruit crops, with 76.3×10^6 t produced on approximately 4.8×10^6 ha in 2012. Turkey is the fourth largest apple-producing country in the world with a production of 2.89×10^6 t in 2012 (http://faostat.fao.org/). Although apple is functionally diploid ($2n = 2x = 34$), the Maloideae are believed to be of allopolyploid origin (Chevreau et al., 1985).

Although apples are grown in many regions of Turkey, approximately 60% of commercial apple production occurs in 5 provinces: Isparta, Karaman, Niğde, Denizli, and Antalya. These provinces are located in the southern part of Central Anatolia and in the northern Mediterranean region. Apples are also commercially grown in the provinces of Kayseri, Çanakkale, Mersin, Konya, and Bursa (http://www.turkstat.gov.tr/).

Most apple cultivars in Turkish commercial orchards are exotic, with local cultivars having very limited production because of their low yields. Nevertheless, local cultivars have various characteristics that make them attractive for breeding purposes. One such example is Amasya, which has a pleasing taste and flavor and other superior fruit quality characteristics. Although fruit size and alternate-bearing characteristics of Amasya limit its commercialization, this cultivar is a very good genetic resource for breeding and genetic studies.

Genetic improvement of apple by marker-assisted selection requires development of transferable molecular markers, construction of genetic maps, and association of molecular markers with quantitative trait loci (QTLs) and major genes of economic and horticultural importance (Wang et al., 2012). Linkage maps are usually constructed using dominant markers, such as random-amplified polymorphic DNA (RAPD), amplified fragment length polymorphism, and codominant simple sequence repeat (SSR, or microsatellite) markers. Because of their reproducibility, transferability, and multiallelic nature,

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many SSR markers have been developed in apple (Guilford et al., 1997; Gianfranceschi et al., 1998; Hokanson et al., 1998; Liebhard et al., 2002; Hemmat et al., 2003; Silfverberg-Dilworth et al., 2006; Han et al., 2009). Furthermore, many apple SSRs can also be used in *Prunus*, *Sorbus*, and *Pyrus* members along with apples of the subfamily Maloideae (Testolin et al., 2000; Oddou-Muratorio et al., 2001; Yamamoto et al., 2002a, 2002b; Fernández-Fernández et al., 2006; Inoue et al., 2007).

The first genetic maps of apple were published by Hemmat et al. (1994), Conner et al. (1997), Seglias and Gessler (1997), and Maliepaard et al. (1998). These maps, however, largely consist of isozyme, restriction fragment length polymorphism, and RAPD markers, which are not easily transferred to the genetic maps of other cultivars. The codominant mode of inheritance of microsatellites not only simplifies the analysis but also facilitates the transfer of markers between genetic maps derived from different crosses. Reference maps based on a backbone of multiallelic SSR markers have been constructed in several studies (Liebhard et al., 2003; Silfverberg-Dilworth et al., 2006; Han et al., 2011; Wang et al., 2012). SSR-based reference maps are good sources for the construction of a genetic map framework for a given cultivar.

In order to study QTLs for fruit quality characteristics such as flavor components in Amasya, a linkage map was needed for this cultivar. Therefore, a genetic linkage map was constructed based on SSR markers of a cross between Amasya (clone Kaşel-41) and Williams' Pride apple cultivars. Using a large number of common SSR markers, an apple reference genetic map was also generated.

2. Materials and methods

2.1. Plant materials and DNA extraction

Ninety-six F_1 individuals derived from a cross between apple cultivars Kaşel-41 and Williams' Pride were used for the construction of a linkage map. The controlled cross was made in 2008 at the Eğirdir Fruit Research Station in Isparta Province, Turkey. Kaşel-41 is a clonal selection from the cultivar Amasya (Eltez et al., 1983). Two replicates each of F_1 plants and their parents on M9 dwarf rootstock were planted in 3 different locations: the Eğirdir Fruit Research Station in Isparta Province, the Apricot Research Station in Malatya Province, and the Black Sea Agricultural Research Institute in Samsun Province.

DNA extractions from F_1 individuals and parents were conducted according to the CTAB-based protocol (Doyle and Doyle, 1990) with minor modifications (Kafkas et al., 2006). DNA concentrations were measured using a Qubit fluorometer (Invitrogen, USA) or were estimated by comparing band intensities with those of known concentrations of λ DNA after 0.8% agarose gel electrophoresis and ethidium bromide staining. DNA samples were then diluted to a concentration of 10 ng/µL for SSR-PCR reactions.

2.2. SSR-PCR analysis

A total of 699 SSR primer pairs from various genera of Rosaceae were used in this study. The list of tested SSR primer pairs with their sources and acronyms is given in Table 1. Before testing primer pairs for segregation, their optimum annealing temperatures were determined by gradient PCR using DNAs of the parents. The primer pairs were then screened for segregation in 12 individuals and the parents.

Table 1. SSR primer pairs used in Kaşel-41 × Willams' Pride F1 population in this study.

Reference	Origin	Acronyms	Markers
Guilford et al. (1997)	*Malus pumila*	NZ	14
Hokanson et al. (1998)	*Malus pumila*	GD	8
Testolin et al. (2000)	*Prunus persica*	UDP	1
Oddou-Muratorio et al. (2001)	*Sorbus torminalis*	MSS	9
Yamamoto et al. (2002b)	*Pyrus pyrifolia - Pyrus communis*	BG, K, HG	9
Yamamoto et al. (2002a)	*Pyrus pyrifolia*	NH, NB	15
Liebhard et al. (2002) and Gianfranceschi et al. (1998)	*Malus pumila*	CH, MS	146
Hemmat et al. (2003)	*Malus pumila*	GD	10
Fernández-Fernández et al. (2006)	*Pyrus communis*	EM	19
Silfverberg-Dilworth et al. (2006)	*Malus pumila*	Hi, CN, AJ, AF, AU, U, Z	148
Inoue et al. (2007)	*Pyrus pyrifolia*	IPPN	14
Han et al. (2009)	*Malus pumila*	BAC	187
Han et al. (2011)	*Malus pumila*	CTG, CN	119
Total			699

SSR-PCR was carried out using a three-primer strategy according to Schuelke (2000) with some modifications. PCR reactions were performed in 12.5-µL total volumes containing 20 ng of DNA; 75 mM Tris-HCl (pH 8.8); 20 mM $(NH_4)_2SO_4$; 2.0 mM $MgCl_2$; 0.01% Tween 20; 200 µM of each dNTP; 10 nM 5′ M13-tailed forward primer; 200 nM reverse primer; 200 nM universal M13-tailed primer (5′-TGTAAAACGACGGCCAGT-3′) labeled with either FAM, VIC, NED, or PET dye; and 0.6 U of Hotstart Taq DNA polymerase.

Amplification was performed in two steps as follows: first, initial denaturation at 94 °C for 3 min followed by 28 cycles at 94 °C for 30 s, 50–60 °C for 45 s, and 72 °C for 60 s; and second, 10 cycles at 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 60 s, followed by a final extension at 72 °C for 10 min. After completion of the PCRs, the reaction mixtures were denatured by mixing together 0.5 µL of amplified product, 0.2 µL of size standard, and 9.8 µL of Hi-Di formamide. Denatured samples were subjected to capillary electrophoresis on an ABI 3130xl genetic analyzer (Applied Biosystems, USA) using a 36-cm capillary array with POP7 as the matrix (Applied Biosystems). The fragments were resolved using version 3.0 of the ABI data collection software, and SSR fragment analysis was performed with ABI GeneScan Analysis version 4.0 software. For some SSR primer pairs, the electrophoretic patterns could not be easily scored; in these cases, dominant scoring was conducted instead. Dominantly scored loci were named according to their allele sizes.

2.3. Sequence-characterized amplified region analysis

Sequence-characterized amplified region (SCAR) markers AL07 and AM19, developed by Tartarini et al. (1999) for scab resistance, were used in the linkage mapping study. The PCR and electrophoresis conditions used were according to these authors.

2.4. Construction of genetic linkage maps

Linkage maps were constructed for each parent independently using a double pseudo-testcross mapping strategy (Grattapaglia and Sederoff, 1994). A consensus map was also constructed using all the segregating markers. In addition to parental and consensus maps, a reference map was built with common markers. Marker orders in the reference map were used as a fixed order in Kaşel-41 and Williams' Pride genetic maps as well as in the consensus map.

Linkage analysis was performed using JoinMap 4.1 (Van Ooijen, 2011), with segregation data coded as indicated by the software. The segregating markers were tested for goodness of fit to the hypothesized Mendelian ratios by the chi-square test in each locus analysis module of JoinMap (Van Ooijen, 2011). To determine linkage groups based on recombination frequency, logarithm of odds (LOD) scores were calculated based on G^2 statistics. During the JoinMap mapping process, a few markers were excluded from the final maps because their linkage phases could not be determined. Markers were also removed when their distorted segregation conflicted with the segregation pattern of neighboring markers.

For all maps, a LOD score of 8.0 was initially set as the linkage threshold to determine markers in the same linkage group, with standard JoinMap parameter settings also applied. We used Kosambi's mapping function (Kosambi, 1994) to convert recombination frequencies into map linkage distances in centimorgans. The regression mapping algorithm was used for map construction. Linkage group numbering was assigned in accordance with the published maps with common SSR markers. Map presentation was carried out using MapChart 2.2 for Windows (Voorrips, 2002).

3. Results

3.1. Microsatellite segregation

Out of 699 SSR primer pairs tested by gradient PCR, 91 failed to amplify DNA. The remaining 608 SSR primer pairs were screened for segregation using parental and 12 F_1 DNAs. While 191 SSR markers were found to be monomorphic, 417 had different segregation patterns. The 417 segregating primer pairs produced a total of 438 markers. Of these, 381 markers were scored codominantly and 36 were scored dominantly. Among codominantly segregating SSR primer pairs, 9 displayed segregation at 2 loci (denoted with the suffixes x and y). The segregation types of the 390 codominantly segregating markers were as follows: ab × cd (128 markers), ef × eg (79 markers), hk × hk (23 markers), lm × ll (80 markers), and nn × np (80 markers). A total of 48 markers were produced from the 36 dominantly segregating primer pairs; 25 of these markers exhibited lm × ll segregation and 23 markers were of the nn × np segregation type. Overall, 128 ab × cd, 79 ef × eg, 23 hk × hk, 105 lm × ll, and 103 nn × np markers were identified. The SCAR marker AL07 linked to the *Vf* gene was of the nn × np segregation type, indicating that Kaşel-41 is sensitive to scab, whereas Williams' Pride is resistant.

3.2. Linkage map construction with common markers

Before constructing consensus and parental maps, a reference map with common markers was generated (Figure 1). Among the 230 common markers used in the linkage mapping, 207 were mapped: 118 ab × cd markers, 70 ef × eg markers, and 19 hk × hk markers distributed along 17 linkage groups (Table 2). The total map length was 1351 cM, with a 6.5 cM average distance between markers. Lengths of linkage groups ranged from 47.4 cM in LG2 to 120.9 cM in LG15. The average linkage-group length was 79.5 cM, and the average number of markers per linkage group was 12.2. LG15 had the highest number

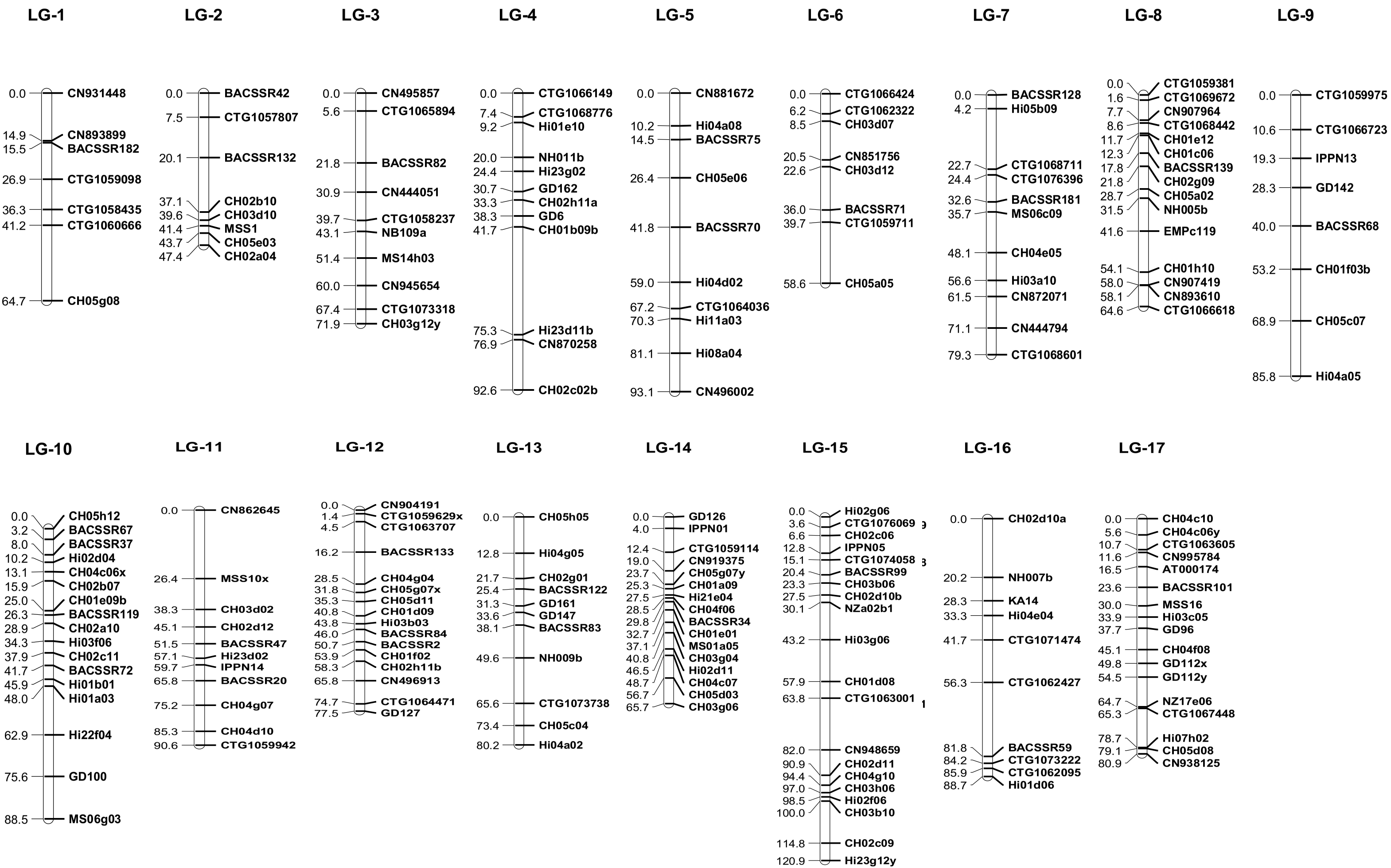

Figure 1. Reference genetic map of apple. The map was generated by 207 common SSR markers using Kaşel-41 × Williams' Pride F1 population.

Table 2. Summary of mapped common SSR markers in Kaşel-41 × Williams' Pride F1 population in apple.

LG	Segregation type			Markers	Length (cM)	Average marker distance (cM)
	ab × cd	ef × eg	hk × hk			
1	2	3	2	7	64.7	9.2
2	6	-	2	8	47.4	5.9
3	7	3	-	10	71.9	7.1
4	6	5	1	12	92.6	7.7
5	5	3	2	10	93.1	9.3
6	4	3	1	8	58.6	7.3
7	2	8	1	11	79.3	7.2
8	11	4	-	15	64.6	4.3
9	3	5	-	8	85.8	10.7
10	10	6	1	17	88.5	5.2
11	8	3	-	11	90.6	8.2
12	10	4	2	16	77.5	4.8
13	5	5	1	11	80.2	7.2
14	13	2	1	16	65.7	4.1
15	13	6	1	20	120.9	6.0
16	5	2	3	10	88.7	8.8
17	8	8	1	17	80.9	4.7
Total	118	70	19	207	1351.0	-
Average	6.9	4.1	1.1	12.2	79.5	6.5

of common markers (20), while LG1 had the lowest (7). Linkage-group average marker distance varied from 4.1 cM in LG14 to 10.7 cM in LG9.

3.3. Construction of parental and consensus maps

Independent linkage maps were constructed for each parent using a double pseudo-testcross mapping strategy. A consensus map was also constructed with all markers. Parental and consensus maps are shown in Figure 2. The marker order in the reference map generated in this study was used to construct parental and consensus maps. An overview of marker segregation types and average number of markers in the three linkage maps is presented in Table 3.

A total of 300 markers were mapped in the maternal parent, Kaşel-41, with an average of 17.7 markers per linkage group, a total map length of 1424.9 cM, and a mean marker density of 4.75 cM. The longest linkage group was LG15, with 23 markers encompassing 128.0 cM; the shortest linkage group was LG8, which comprised 21 markers spanning 61.6 cM. The highest average marker density, 2.93 cM, was in LG8, whereas the lowest density was 7.41 cM in LG16. The largest gap (6.23 cM) was between markers CH01b09b and Hi23d11b in LG4.

The Williams' Pride genetic map comprised 300 SSRs and one SCAR marker, with an average of 17.7 markers per linkage group. Total map length was 1436.4 cM, with a mean marker density of 4.77 cM. The longest linkage group was LG15, with 32 markers spanning 130.2 cM; the shortest linkage group was LG2, which had only 9 markers spanning 56.6 cM. The highest average marker density was in LG12, with 3.45 cM, whereas the lowest was 8.07 cM in LG5. Two large gaps were identified: a 33.6-cM gap between markers CH01d07 and Hi23d11b in LG4, and a 26.5-cM gap between markers CN862645 and MSS10x in LG11 (Figure 2).

The consensus genetic map had a 1476.4-cM total length with a mean marker density of 3.82 cM. The map included 396 markers, with an average of 23.3 markers per linkage group. The longest linkage group was LG15, which comprised 35 markers with a 135.1-cM length. The shortest linkage group was LG8, with 27 markers measuring 64.1 cM. The highest average marker density, 2.37 cM, was in LG8; the lowest was 5.04 cM in LG9. No gaps larger than 20 cM were detected in the consensus map. The largest gap, 10.5 cM, was between markers Hi07d11-239 and MSS10x in LG11.

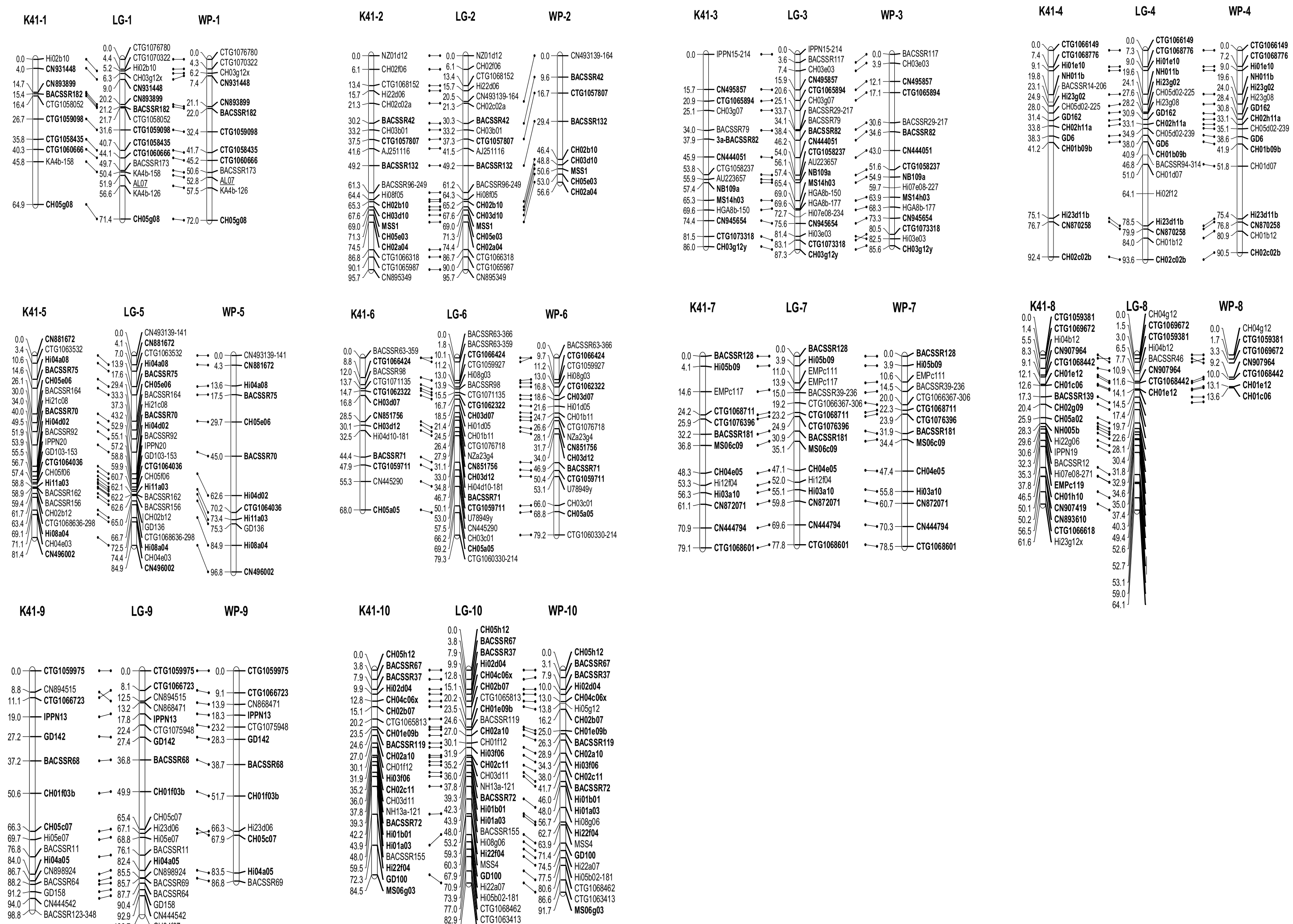

Figure 2. The consensus and parental maps in Kaşel-41 × Williams' Pride F1 population. The consensus map is shown in the center while the parental maps are presented on the left and right sides.

Table 3. Mapped SSR markers with segregation types in Kaşel-41, Williams Pride, and consensus maps.

Segregation type	Kaşel-41	Consensus	Williams' Pride
ab × cd	118	119	118
ef × eg	70	69	70
hk × hk	19	19	19
lm × ll	74	75	-
nn × np	-	75	74
Dominant-lm × ll	19	21	-
Dominant-nn × np	-	18	21
Total markers	300	396	302
Average marker	17.64	23.29	17.76
Map length (cM)	1424.9	1476.4	1436.6
Average marker density (cM)	4.75	3.73	4.76

4. Discussion

4.1. Microsatellite segregation

In this study, 699 SSR primer pairs were analyzed, 417 of which had different segregation patterns. Among the segregating SSR primer pairs, 9 exhibited segregation at 2 loci, whereas 6 (Hi23g12, MSS10, BAC SSR187, CTG1059629, U78949, and GD112) amplified single loci in maps constructed by Silfverberg-Dilworth et al. (2006), Han et al. (2011), and Wang et al. (2012).

Among the 417 segregating SSR markers, 108 were expressed as sequence tag SSRs, 57 were bacterial artificial chromosome SSRs (BAC SSRs), and the remainder were genomic SSRs. Han et al. (2011) found 32 (17.1%) of 187 BAC SSR primers to be polymorphic between apple cultivars Co-op 16 and Co-op 17; in our study, 67 BAC SSR markers were polymorphic between Kaşel-41 and Williams' Pride, with 57 of them subsequently mapped. These results demonstrate the importance of parental selection in genetic mapping studies.

A total of 207 common SSR markers were mapped in this study. Using a Fiesta × Discovery F_1 population, Silfverberg-Dilworth et al. (2006) mapped 86 common markers in the first reference genetic map of apple. Fernández-Fernández et al. (2008), Han et al. (2011), and Wang et al. (2012) subsequently mapped 165, approximately 163, and 83 common markers in Fiesta × Totem, Co-op 16 × Co-op 17, and Royal Gala × PI613988 F_1 populations, respectively.

The SCAR marker AL07, linked to scab resistance in apple, exhibited an nn × np segregation type. These data confirm that Amasya (clone Kaşel-41) is sensitive to scab, whereas Williams' Pride is resistant. The scab resistance of Williams' Pride was also demonstrated in a previous study (Janick et al., 1988).

4.2. Linkage map construction with common markers

Reference genetic maps are very important for genetic mapping of individual species, especially heterozygous species where F_1 plants are used, as enough common markers must exist between the maps constructed for each parent to allow the maps to be combined. The first detailed apple reference genetic map was published by Silfverberg-Dilworth et al. (2006), who mapped 86 common SSR markers. Thereafter, Han et al. (2011) and Wang et al. (2012) mapped 163 and 83 common SSR markers in apple, respectively.

To build an improved apple reference map, the previously published reference genetic maps were used as a guide in this study. The new reference genetic map was constructed based on 207 common markers distributed along 17 linkage groups, a higher number of markers than in other genetic mapping studies in apple. The highest number of markers, 20, was in LG15, which was also the longest linkage group, covering 120.9 cM. Previous studies (Silfverberg-Dilworth et al., 2006; Han et al., 2011; Wang et al., 2012) have also indicated that LG15 is the longest linkage group in apple.

The average number of markers per linkage group observed in this study, 12.2, was higher than that uncovered in previous studies performed by Silfverberg-Dilworth et al. (2006), Han et al. (2011), and Wang et al. (2012), who reported values of 5.1, 9.6, and 4.9, respectively. Our reference map with common markers is also denser (6.5 cM) and longer (1351 cM) than the other published maps. The map constructed with common markers in this study

can be used as a reference map in future studies of apple, such as genetic diversity assessment, genetic mapping, and molecular breeding applications.

4.3. Construction of parental and consensus maps

Kaşel-41 and Williams' Pride genetic maps were constructed with 300 SSR markers, which included 207 markers common to both cultivars, 93 specific to Kaşel-41, and 93 specific to Williams' Pride. The Williams' Pride genetic map also included a SCAR marker. Silfverberg-Dilworth et al. (2006) mapped 99 and 115 SSR markers in apple cultivars Fiesta and Discovery, respectively. Han et al. (2011) mapped 279 and 272 SSR markers in Co-op 16 and Co-op 17, respectively, and Wang et al. (2012) mapped 190 SSR markers in Royal Gala. The Kaşel-41 and Williams' Pride genetic maps, both with 300 SSR markers, have more SSR markers than any apple genetic maps reported to date.

Although most markers in this study were located in the same linkage groups as in previous studies, several were not. These positional differences are not easily explained. However, an integration of physical and genetic maps in apple by Han et al. (2011) revealed duplication of linkage group pairs such as LG1-LG7, LG2-LG15, LG3-LG11, LG4-LG12, LG5-LG10, LG6-LG14, LG8-LG15, LG9-LG17, and LG13-LG16. Such duplication may be partly responsible for positional differences between different mapping studies. Consistent with these observations, for instance, marker CH05a02 was mapped to LG8 in this study, whereas Liebhard et al. (2003) mapped it to LG15. Similarly, marker CH02c06 was located in LG15 in this study, although it was mapped to LG2 in different studies (Liebhard et al., 2003; Silfverberg-Dilworth et al., 2006; Fernández-Fernández et al., 2008). Although CH01h02 was mapped to LG9 by Liebhard et al. (2003) and Fernández-Fernández et al. (2008), it was located in LG17 in this study.

In contrast to the above examples, some positional differences were inconsistent with duplication patterns reported by Han et al. (2011). For example, the Hi02b10 marker located in LG1 in this study was previously mapped to LG16 by Silfverberg-Dilworth et al. (2006). In this case, the primer pair may have amplified multiple loci, with PCR products of different sizes obtained in the two studies.

An analysis of inheritance of fruit ring rot resistance in apple by Zhuang et al. (2011) potentially linked markers CH01e01 (LG14) and CH02c02b (LG4) to the susceptible locus of the Ls1 pathogen. These markers were located in the same linkage groups in this study. Similarly, markers CH03a03 and CH05d11 have been linked to a locus susceptible to pathogen isolate LW048 and mapped to LG14 and LG12, respectively (Zhuang et al., 2011). These markers also mapped to LG14 and LG12 in this study.

In addition, the resistance gene *Rvp1* (for resistance to *Venturia pirina*), previously reported on LG2 of the pear genome near microsatellite marker CH02b10 (Bouvier et al., 2012), was mapped in this study to a similar location in apple. In apple, this region is known to contain a cluster of scab resistance genes and thus corresponds to the first functional synteny identified for scab resistance between apple and pear. This finding demonstrates that the SSR markers mapped in this study may also assist genetic mapping studies in pear, which has a close relationship with apple.

In this study, the positions of 57 SSR loci that were previously unmapped in apple were identified. Five markers developed by Inoue et al. (2007) from *Pyrus pyrifolia* (IPPN01, IPPN08, IPPN09, IPPN13, and IPPN20) were located on LG14, LG15, LG15, LG9, and LG5, respectively. The marker EMPc110 from *P. communis* developed by Fernández-Fernández et al. (2006) was located on LG13. Markers NB109a, NH13a, NH30a, NH015a, and Ka14 developed by Yamamoto et al. (2002a, 2002b) were located on LG3, LG10, LG11, LG17, and LG16 respectively. The marker MSS1 developed from *Sorbus torminalis* was located on LG2 (Oddou-Muratorio et al., 2001). The remaining 45 SSR markers, developed from *Malus pumila*, were located along 17 linkage groups. The newly mapped SSRs have approximately identical marker orders on both parental and consensus maps, thus allowing exploration of recombination frequencies in equivalent genetic regions between male and female gametes.

In this study, 400 SSR markers were mapped using an F_1 population between cultivars Kaşel-41 and Williams' Pride. The highest number of common SSR markers reported to date was generated, enabling the construction of a denser reference map in apple. This reference map will aid further genetic mapping and population genetic studies in apple and closely related species. Future incorporation of additional markers, such as SNPs, will increase map saturation and yield a more comprehensive genetic map. Finally, evaluation of flavor compounds and other phenotypic traits of Kaşel-41 × Williams' Pride F_1 populations from the three ecologically different locations will facilitate linkage between molecular markers and phenotypic traits. Such linkage may aid in the cloning of major genes and QTLs controlling economically important traits in apple.

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