DNA Fingerprinting of Closely Related Cultivars of Sweet Cherry

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ABSTRACT. Simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers were evaluated in an effort to reliably DNA fingerprint sweet cherry (Prunus avium L.) cultivars and advanced selections from the breeding program at the Summerland Research and Development Center (Summerland, BC, Canada). SSR markers were found that differentiated the 35 cultivars and selections tested. However, groups of cultivars closely related to the parental cultivars, Lapins and Sweetheart, were differentiated by only a few SSR markers each. These last few markers were discovered by specifically screening within these small groups of cultivars and the resulting markers had lower discriminating power (D) statistics within the full set of 35 cultivars and selections. To further characterize the differences in one of these closely related groups, SNP markers were identified in the cultivar Sweetheart and an analysis was made of how these markers segregated into three of its open-pollinated progeny. Large blocks of the ‘Sweetheart’ genome (34%) did not contain informative SNP markers, which was consistent with its ancestry where the cultivar Van is both a parent and grandparent. The three progeny cultivars differed from ‘Sweetheart’ at 14%, 31%, and 29% of the 3011 SNP positions tested. These were located in blocks of linked haplotypes covering from 2.5 to 20 million bps each and were distinct for the three cultivars. The cultivar Staccato*, which required the most effort for SSR marker discrimination, also had the lowest number of SNP position differences from ‘Sweetheart’ (14%). These informative SNP markers were located in only five small regions of the sweet cherry genome, which also contained the discriminating SSR markers and provides an explanation for the difficulty of locating SSR markers for this cultivar. In addition to clearly differentiating these cultivars, this SNP analysis shows the level of variation expected within this closely related group.

An international breeding program has an ongoing requirement to reliably identify their cultivars to maintain correct material during propagation and for enforcement of plant breeders’ rights. The sweet cherry breeding program from the Summerland Research and Development Center of Agriculture-Agri-Food Canada (SuRDC, Summerland, BC, Canada) and their official licensing organization, Summerland Varieties Corporation, continue to identify new cultivars, which require the precision of identification that is available with DNA marker technologies. Although a similar study had been carried out with amplified fragment length polymorphism (AFLP) markers (Zhou et al., 2002) it was determined that the level of hands-on expertise required to run and evaluate these markers was too high for occasional use, also noted by Noli et al. (2013). In the three decades since DNA markers were first introduced, there has been rapid development of marker technologies, particularly with the advent of DNA amplification in polymerase chain reaction (PCR). When this project was started, SSR markers were the marker of choice (Nybom et al., 2014), with extensive sets of markers developed for sweet cherry and other closely related Prunus L. species (Jung et al., 2014; Olmstead et al., 2008). Previous studies with sweet cherry showed that a few, highly informative, markers were sufficient to differentiate a wide range of cultivars (Dirlewanger et al., 2002; Fernandez i Marti et al., 2012; Pedersen, 2006; Wunsch and Hormaza, 2004). This method was, therefore, tested on 35 cultivars (i.e., named cultivars and advanced selections) from the SuRDC program, some of which were difficult to differentiate by AFLP (Zhou et al., 2002). Many of these cultivars are currently licensed, or are advanced selections with the potential for licensing, and are expected to be propagated and planted worldwide, making a reproducible means of identification at the DNA level an essential tool.

Materials and Methods

Sample preparation. Reference trees for the 35 sweet cherry cultivars and selections in Table 2 were identified using the records of the sweet cherry breeding program and located
within the orchards of the SuRDC. Young expanding leaf tissue was collected from mature sweet cherry trees in spring and samples were frozen and stored at −80 °C until extraction. DNA extraction was by the method of Zhou et al. (2002) and RNA extraction was by a modification of the method of Fils-Lycaon et al. (1996). For RNA extraction, 0.5 g of frozen powdered tissue was quickly mixed with 1.2 mL of extraction buffer (0.2 mM glycine, 0.1 mM disodium phosphate, 0.6 mM sodium chloride, pH 9.5); 66 μL of 2-mercaptoethanol; 66 μL of 20% (w/v) sodium dodecysulfate; and 1 mL of phenol (equilibrated greater than pH 7.6 with Tris-HCl). After centrifugation, the clear aqueous layer was removed to a clean tube and ethanol added to a final concentration of 30% (v/v). This RNA solution was pipetted into a disposable plastic column (Poly-Prep; Bio-Rad, Hercules, CA) containing a bed of ≈200 mg of CC41 cellulose (Whatman, Maidstone, England) suspended in 1X STE (100 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA) and 30% ethanol. The column was attached to a vacuum manifold and washed with 40 mL of 1X STE-30% ethanol to remove DNA and contaminants. The RNA was eluted from the column by centrifugation with three washes of RNase-free water (550 μL total); cellulose fines were removed by centrifugation and the RNA recovered by ethanol precipitation.

SSR analysis. SSR primer sequences were as referenced from Olmstead et al. (2008) and the Genome Database for Rosaceae (GDR; Jung et al., 2014). PCR reactions, in a total volume of 25 μL, contained 2.5 mM MgCl₂, 0.3 mM each of forward and reverse primer, 200 μM dNTPs, 0.5 U of AmpliTaq® Gold DNA polymerase (Applied Biosystems, Foster City, CA), and 1X PCR Buffer II (supplied with the enzyme). Dilutions of genomic DNA were optimized for each primer pair with input DNA ranging from 1 to 50 ng per reaction. Thermocycling conditions were 95 °C for 10 min; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. SSR reactions were electrophoresed on polyacrylamide gels and silver stained following the detailed protocol of George and Regalado (2003). SSR statistics were evaluated in a computer spreadsheet (Excel; Microsoft, Redmond, WA) from the following equations, with \( P_i \) or \( p_j \) as the frequency of allele \( i \) or \( j \), \( g_i \) as the frequency of genotype (or pattern) \( i \), and \( N \) as total number of compared cultivars: gene diversity, \( H_e = \sum p_i^2 \), Nei (1973); direct count (observed) heterozygosity, \( H_o = \) number of samples with heterozygous genotypes/total number of samples; probability of identity, \( P_{ID} = \sum p_i^4 + \sum \left(2p_i p_j\right)^2 \), with \( i < j \) in the second term, Waits et al. (2001); confusion probability (\( C_j \)), \( C_j = \sum g_i^2 \left(\frac{N_2 - 1}{N - 1}\right) \), Tessier et al. (1999); discriminating power, \( D_j = 1 - C_j \) or \( D_L = DP = 1 - \sum g_i^2 \), Tessier et al. (1999), Kloosterman et al. (1993); number of nondifferentiated pairs, \( x_j = C_j \times \frac{N}{2} \), Tessier et al. (1999). The genotype for each cultivar was compared against the other 34 cultivars in this study using macros in Excel. The spreadsheet calculated the differences between each pair of cultivars as either the number of bands or the number of genotypes. Total number of comparisons for the 35 cultivars was 595 (35 choose 2) with each comparison scored as either “same,” with all bands or genotypes identical between the two cultivars or “diff,” with at least one different band or genotype.

Single nucleotide polymorphism analysis. RNA-Seq libraries were prepared from young leaf mRNA of the cultivars Sweetheart and three of its offspring Staccato®, Sovereign™, and Sentennial™ with a TruSeq kit (Illumina, San Diego, CA) according to the manufacturer’s instructions. Two lanes of HiSeq Illumina sequence data were obtained from the BC Cancer Genome Center, Vancouver, BC, producing 200 million \( \times 2 \times 75 \) bp reads from Sweetheart and 65 million reads for each of the other three cultivars. SNP markers were identified de novo in the ‘Sweetheart’ RNA-Seq reads using KissSplice (Sacomoto et al., 2012) with options -s 1 -k51. The sequence surrounding each SNP (a 103-bp KissSplice fragment) was used to map that sequence within the published sweet cherry genome (Shirasawa et al., 2017) using BLAST+ (Camacho et al. (2009)). The RNA-Seq reads for the four cultivars were individually aligned to the sweet cherry genome using BWA MEM (Li and Durbin, 2010). The SNP markers identified in ‘Sweetheart’ were then evaluated for all four cultivars by running their alignments together through samtools mpileup using the -l option to specify SNP locations and bcftools view to determine genotypes (Li, 2011). This SNP set was refined by requiring a) a good match in the sweet cherry transcriptome; b) that the fragment mapped to a unique location in the eight sweet cherry pseudomolecules (i.e., chromosomes); and c) a raw read depth (vcftools “DP” ) above 100. A map of the SNP locations for these cultivars on the sweet cherry pseudomolecules was displayed using a modification of the Bio.Graphics.BasicChromosome module from BioPython Pritchard et al. (2006). The SNP sequences, map locations, and genotypes at these positions for ‘Sweetheart’, ‘Staccato®’, ‘Sovereign™’, and ‘Sentennial™’ have been deposited in the GDR marker database.

Results and Discussion

Making use of the large number of SSR markers previously demonstrated to function in sweet cherry (Jung et al., 2014; Olmstead et al., 2008) we began screening these markers for their ability to differentiate 35 selected cultivars from the SuRDC breeding program. Screening was carried out with small sets of cultivars to evaluate amplification and the resulting fragment sizes and patterns. Selected markers were then assessed on all 35 cultivars, and the three most informative markers (pms3, pms30, and IDP98-022 had the highest number of genotypes, highest DP, lowest IDP, and highest number of differences between cultivars) were sufficient to differentiate 26 of these cultivars (Tables 1 and 2). This is consistent with the results of previous analyses with sweet cherry, where a few highly informative markers were sufficient for discrimination of a broad range of cultivars. However, three groups of closely related cultivars showed no differences with these markers. These groups were: ‘Lapins’ and two of its progeny, ‘Sweetheart’ and two of its progeny, and three ‘Stella’ progeny. Because the difficulty of differentiating the cultivar Sweetheart from its progeny had already been demonstrated (Zhou et al., 2002), additional markers were screened using only eight cultivars, specifically to find differences within these groups. With the addition of three of these markers (BPPCT 014, EMPaS02, and pms67) to the three most informative markers, we were able to differentiate all cultivars except Sweetheart and Staccato®. This included ‘Sovereign™’, ‘Sentennial™’, and ‘SPC243’, which are also open-pollinated ‘Sweetheart’ progeny and were expected to pose a similar
Table 1. Simple sequence repeat markers used for discrimination of 35 sweet cherry cultivars with genome location and marker statistics.

| Marker    | Start position | Allele sizes | Same (no.) | Different (no.) | Genotypes | Alleles (no.) | \( H_e \) | \( H_o \) | \( P_{ID} \) | \( C_j \) | \( D_j \) | DP | \( x_j \) |
|-----------|----------------|--------------|------------|-----------------|-----------|---------------|---------|---------|-------------|-------|-------|-----|--------|
| PMS3      | PAV_r1.0chr4:4,591,257 | 209, 197, 189, 187 | 105        | 490             | 8         | 4             | 0.68    | 0.74    | 0.17        | 0.18  | 0.82  | 0.80 | 105   |
| PMS30     | PAV_r1.0chr3:4,259,372 | 188, 164, 152, 142 | 110        | 485             | 8         | 4             | 0.63    | 0.60    | 0.21        | 0.18  | 0.82  | 0.79 | 110   |
| UDP98-022 | PAV_r1.0chr1:43,105,558 | 106, 104, 95, 91 | 126        | 469             | 7         | 4             | 0.63    | 0.74    | 0.20        | 0.21  | 0.79  | 0.77 | 126   |
| EPPCU0961 | PAV_r1.0chr5:2,645,604 | 149, 147, 145 | 146        | 449             | 6         | 3             | 0.67    | 0.85    | 0.20        | 0.25  | 0.75  | 0.73 | 146   |
| BPPCT 014 | PAV_r1.0chr5:15,080,604 | 193, 191, 189 | 173        | 422             | 5         | 3             | 0.52    | 0.49    | 0.33        | 0.29  | 0.69  | 0.69 | 173   |
| EMMPaS02  | PAV_r1.0chr3:19,949,784 | 147, 145, 144 | 195        | 400             | 6         | 5             | 0.42    | 0.49    | 0.36        | 0.33  | 0.67  | 0.65 | 195   |
| PMS67     | PAV_r1.0chr1:28,938,079 | 158, 154, 149 | 218        | 377             | 5         | 3             | 0.55    | 0.69    | 0.30        | 0.37  | 0.63  | 0.62 | 218   |
| UCD-CH12  | PAV_r1.0chr2:20,954,673 | 196, 179, 175 | 221        | 374             | 5         | 3             | 0.50    | 0.60    | 0.34        | 0.37  | 0.63  | 0.61 | 221   |

*a* Allele sizes for each marker are identified by letter (a–e) and are used in the scoring in Table 2. 
*y* Location in sweet cherry genome assembly pseudomolecules (Shirasawa et al., 2017). 
*x* Number of comparisons between each pair of the 35 cultivars giving the same (or different) genotype [out of 595 = (35 choose 2)].

\( H_e \) = gene diversity; \( H_o \) = observed heterozygosity; \( P_{ID} \) = probability of identity; \( C_j \) = confusion probability; \( D_j \) and DP = discriminating power; \( x_j \) = number of nondifferentiated pairs. 

Equations for these values given in the Materials and Methods.
Table 2. Scoring of simple sequence repeat (SSR) markers for 35 sweet cherry cultivars, with parentage.

| Cultivar         | pms3 | pms30 | UDP98-022 | EPPCU0961 | BPPCT 014 | EMPaS02 | pms67 | UCD-CH12 | Parentage                                                                 |
|------------------|------|-------|-----------|-----------|-----------|----------|-------|-----------|----------------------------------------------------------------------------|
| Bing             | 1    | 2     | 3         | 4         | 5         | 6        | 7     | 8         | 'Black Republican' × 'Napoleon'                                            |
| Summex (Cristalina™) | ad   | ac    | ac        | ab        |            |          |       |           | 'Star' × 'Deacon' (OP) × 'Van'                                           |
| Lapins           | ac   | ac    | ac        | cd        | ab        | e        |       |           | 'Van' × 'Stella'                                                          |
| Sumini (New Moon™) | c    | ac    | ac        | d         | c         | ab       | ce    | ab        | 'Van' × 'Stella'                                                          |
| Newstar          | cd   | bc    | c         | c         | bc        | c         |       |           | 'Van' × 'Stella'                                                          |
| Sumste (Samba™)  | c    | c     | a         | bd        | ac        | e         |       |           | 2S-84-10 × 'Stella' (self) × 'Stella'                                     |
| Sandra Rose      | cd   | ab    | a         | c         | ab        | bc        | be    | ac        | 2C-61-18 × 'Star' (Deacon (OP) × 'Van') × 'Sunburst' × 'Van' (Stella)     |
| Santina          | ad   | bc    | c         | ac        | ab        | bc        |       |           | 'Stella' × 'Summit'                                                       |
| Sumele (Satini™) | cd   | a     | a         | cd        | c         | ab        | e     | c         | 'Lapins' × 2N-39-05 (Van × Stella)                                       |
| SPCL03 (Sentennial™) | ac | a    | ac        | cd        | a         | e         |       |           | 'Sweetheart' × OP                                                         |
| Skeena           | ad   | ad    | ac        | cd        | c         | ab        | ae    | a         | 2N-60-07 × 'Bing' (Stella) × 2N-38-22 (Van × Stella)                      |
| SPCL06 (Sofia™)  | bd   | ac    | ab        | c         | bc        | b         | ae    | ac        | OSC#6 (Brennel × Lambert) × 2S-28-39 (Van × Stella)                       |
| Sunleta (Sonata™) | ac   | a     | a         | ac        | ab        | e         |       |           | 'Lapins' × 2N-39-05 (Van × Stella)                                       |
| 13S-21-01 (Sojourn™) | ac | a    | ac        | c         | a         | ac        | e     | c         | 'Sweetheart' × OP                                                         |
| SPC118           | ad   | c     | ac        | c         | a         | ab        | e     | ac        | 'Stella' × 'Summit'                                                       |
| SPC133           | ad   | bc    | ac        | c         | ab        | ae        | ac    |           | 4A-03-06 × (2C-27-29 × self) × Compact Stella x)                          |
| SPC171           | ad   | cd    | ac        | d         | a         | bc        | ce    | a         | 2N-60-07 × 'Bing' × 'Stella' × 2N-38-22 (Van × Stella)                    |
| SPC175           | ad   | ac    | ac        | ad        | a         | bc        | ae    |           | 'Compact Lambert' × 'Lapins'                                              |
| SPC189           | ad   | ac    | ac        | cd        | c         | ab        | ce    | a         | 'Lapins' × 2N-39-05 (Van × Stella)                                       |
| SPC221           | cd   | c     | ac        | c         | ac        | a         | e     | ac        | 2C-61-18 × 'Star' (Deacon (OP) × 'Van') × Summer Jewel                    |
| SPC232           | ac   | ac    | ac        | cd        | a         | ab        | e     | c         | 'Lapins' × 'Summit'                                                       |
| SPC243           | a    | a     | c         | cd        | ac        | a         | e     | ac        | 'Sunburst' × OP                                                          |
| SPC378           | c    | c     | c         | bd        | ac        | ac        | e     | ac        | 'Stella' × 'Summit' (Van × Stella) × 13S-30-21 (Sandra Rose × self)       |
| SPC381           | c    | c     | a         | cd        | c         | a         | ac    |           | 'Lapins' × 'Sonnet' × (2N-49-02 (Van × Stella) × 2N-63-31 (Bing × Salmo)     |
| 13S-20-09 (Staccato™) | ac | a    | ac        | cd        | ac        | a         | e     | c         | 'Sweetheart' × OP                                                         |
| 13N0739 (Starblush™) | ac | ac   | ab        | cd        | ac        | ab        | ac    | ac        | 2N-63-20 × (Bing × Salmo) × 'Stella'                                        |
| SPC207 (Staveletta™) | bc | ac   | ab        | cd        | ac        | ab        | ac    | ac        | 'Stella' × 2S-84-10 (Stella × self)                                       |
| Stella           | ac   | bc    | ac        | ac        | ab        | ae        | ac    |           | 'Lambert' × JI 2420 (Emperor Francis × Napoleon)                           |
| SPC136 (Suite Note™) | d  | cd    | c         | cd        | ac        | bc        | de    | c         | 2S-36-36 × 4C-16-10 (Van × Compact Lambert) × Stella × Summit              |
| Sumbola          | d    | c     | ac        | bc        | c         | ac        | ce    | ac        | 'Star' × (Deacon (OP) × 2S-41-27 (2C-27-29 × self)                         |
| Sumesi           | ac   | b     | ac        | bc        | ac        | ac        | e     | c         | 'Van' × 2S-22-5 (Stella × self)                                           |
| Summer Jewel     | ac   | c     | c         | bc        | c         | ab        | ce    | ac        | 2C-61-18 × 'Star' (Deacon (OP) × 'Van') × 2S-28-30 (Van × Stella)         |
| Summit           | d    | c     | ac        | c         | ac        | bc        | e     | c         | 'Van' × 'Sam' [V16140 (Windsor (OP) × OP]                                  |
| Sumtare (Sweetheart™) | ac | a    | ac        | cd        | ac        | ac        | e     | ac        | 'Van' × 'Newstar' (Van × Stella)                                          |
| Van              | cd   | ac    | ac        | cd        | c         | ac        | ce    | ac        | 'Empress Eugenie' × OP                                                    |

Each letter in the score table refers to the allele of a given size for that SSR marker from Table 1. OP = open-pollinated.

'Lambert' (Napoleon × Black Heart); 'Compact Lambert' = X-ray treated 'Lambert' (Lapins, 1963).

'Compact Stella' = X-ray treated 'Stella' (Lapins, 1974).
The genotype for 'Lapins' in Table 2 is consistent at all loci for that 'Lapins' is more likely the parent (with 'Van') for this cultivar. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion associated with these crosses. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion associated with these crosses. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion that 'Lapins' is more likely the parent (with 'Van') for this cultivar. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion that 'Lapins' is more likely the parent (with 'Van') for this cultivar. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion that 'Lapins' is more likely the parent (with 'Van') for this cultivar. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion that 'Lapins' is more likely the parent (with 'Van') for this cultivar. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion that 'Lapins' is more likely the parent (with 'Van') for this cultivar. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion that 'Lapins' is more likely the parent (with 'Van') for this cultivar. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion that 'Lapins' is more likely the parent (with 'Van') for this cultivar. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion that 'Lapins' is more likely the parent (with 'Van') for this cultivar. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion that 'Lapins' is more likely the parent (with 'Van') for this cultivar. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion that 'Lapins' is more likely the parent (with 'Van') for this cultivar. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion that 'Lapins' is more likely the parent (with 'Van') for this cultivar. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion that 'Lapins' is more likely the parent (with 'Van') for this cultivar. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion that 'Lapins' is more likely the parent (with 'Van') for this cultivar. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion that 'Lapins' is more likely the parent (with 'Van') for this cultivar. The parentage of 'Sweetheart' has been previously questioned (Cai et al., 2017) with the suggestion that 'Lapins' is more likely the parent (with 'Van') for this cultivar.
in finding SSR markers that were able to distinguish between ‘Sweetheart’ and ‘Staccato®’ because there are only five small blocks in the genome where ‘Staccato®’ differs from ‘Sweetheart’ (located at the bottom of chromosomes 2, 3, and 7 and the top of chromosomes 4 and 5). ‘Staccato®’ has a considerably reduced number of homozygous loci even compared with its two sibs. All three of these cultivars are the end product of selection during the breeding process so their genotypes could be far removed from a random segregation of these sites. Large regions without SNP markers in ‘Sweetheart’ were found on the top of chromosomes 1 and 3; the middle of chromosomes 2, 5, and 7; and the bottom of chromosome 4. These regions were found in each of the progeny and probably indicate inheritance of haplotype blocks from the cultivar ‘Van’ which is both a parent and grandparent of ‘Sweetheart’. The only location where all three progeny differ from their parent is at the bottom of chromosome 2. This region has been shown to contain several major quantitative trait loci for fruit traits (Cai et al., 2017), which would be of particular interest to the breeding program. Interestingly, the progeny differ from each other at that location with ‘Staccato®’ and ‘Sovereign™’ homozygous for one set of phased alleles whereas ‘Sentennial™’ bears only the other allele in each location. Although the homozygous blocks were distinct, some intervening SNP markers remained after all refining criteria were applied. These were examined individually in alignments of all available reads and most appeared to be true polymorphisms. Although the possibility of unique noncrossover recombination events were considered, it is also possible that these interruptions in the sequence may instead be related to some uncertainty of assignment of contigs in this first draft of the sweet cherry genome.

At the current time neither of the two methods used in this study would be recommended for discovery of markers for DNA fingerprinting of highly related individuals. Although SSR markers can be used to discriminate sweet cherry cultivars as previously suggested, finding markers that differentiate closely related cultivars, such as the Lapins and ‘Sweetheart’, groups required extensive screening. In addition, this specific combination of markers would be expected to be useful only for these groups. The use of RNA-Seq for genotyping is more expensive than other reduced representation methods and although effective is not recommended for general use. Until sequencing becomes inexpensive enough for routine production of full genomes, some form of reduced representation sequencing would be appropriate for this analysis. Data from the RosBREED cherry SNP array (Hewitt et al., 2017; Peace et al., 2012) and preliminary analysis using restriction site–associated DNA sequences [RADseq (Elshire et al., 2011); data not shown] provided confirming genotypes for the segregation of SNP markers into the ‘Sweetheart’ progeny. The RosBREED cherry SNP array was not originally considered in our analysis, as the broad set of cultivars used for development of the SNP markers was expected to provide too few markers for the closely related cultivars in this study. The data from supplemental file ‘mmc’ from Hewitt et al. (2017), however, gave 451 loci where ‘Sweetheart’ was homozygous, which is significantly better than the 40 heterozygous SSR loci in the present study. Of these ‘Sweetheart’ SNP markers, 72 (16%) were homozygous in ‘Staccato®’ which is consistent with the RNA-seq SNP analysis. Because we are considering only the ‘Sweetheart’ SNP markers for segregation into its progeny this result would not be expected to show an ascertainment bias such as suggested by Hewitt et al. (2017) when the results of the entire SNP array were considered. Both SNP array and RADseq analyses show unique regions of homozygosity in ‘Staccato®’ (data not shown); which were the same as those found for ‘Staccato®’ in the RNA-Seq analysis of Fig. 1.

Finally, the problem remains of how to use this data for the routine analysis of sweet cherry cultivars. Two recurring needs have been the testing for trueness-to-type during propagation and distribution; and comparing potential new cultivars with previously licensed material for the initial investigation for the enforcement of breeders’ rights. Both requirements often involve only a few samples at a time for which NextGen Sequencing and SNP array methods are not recommended because they rely on multiplexing or multiple samples, respectively, to reduce cost per sample. Instead, a small representative set of SNP markers can be chosen for which SNP-spanning PCR products can be analyzed in single samples. Techniques compatible with this approach include high-resolution melting (Wittwer et al., 2003), commercial assays (e.g., Semagn et al., 2014), and oligo ligation assays (Macdonald et al., 2005). To maximize the applicability of markers to the closely related cultivars in the ‘Sweetheart’ group, the SNP locations can include the four small regions showing differences between ‘Sweetheart’ and ‘Staccato®’. A set of 16 markers chosen on opposite ends of the eight sweet cherry chromosomes, and therefore expected to be inherited independently, would increase the information content and would be sufficient to differentiate all 35 cultivars in this study, including the closely related cultivars in the ‘Sweetheart’ and ‘Lapins’ groups.

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### Supplemental Table 1. Simple sequence repeat (SSR) markers tested for amplification in sweet cherry.

| ‘Sweetheart’ heterozygous | ‘Sweetheart’ homozygous | Poor amplification | Complex | Unscorable |
|--------------------------|-------------------------|--------------------|---------|------------|
| AMPA101                  | AMPA093                 | BPPCT 005          | BPPCT 010 | CPSCT019   |
| BPPCT 002                | AMPA110                 | BPPCT 006          | BPPCT 013 | EMPA014    |
| BPPCT 014                | AMPA112                 | BPPCT 034          | BPPCT 035 | UDA-005    |
| BPPCT 039                | BPPCT 008               | BPPCT026           | EPDCU5100 | UDA-037    |
| BPPCT 040                | BPPCT 009               | CPPCT3             | EPDCU4726 |            |
| BPPCT037                | BPPCT 016               | CPPCT5             | MA023a   |            |
| CPDCT017                | BPPCT 024               | EMPA005            | pchcms1  |            |
| CPDCT022                | BPPCT 042               | EPDCU3516          |         |            |
| CPDCT028                | CPDCT037                | MA014a             |         |            |
| CPPCT29                 | CPDCT045                | MA066a             |         |            |
| CPPCT33                 | CPPCT017                | PceGA25            |         |            |
| CPSCT027/AY426212       | CPPCT16                 | UDA-013            |         |            |
| EMPA015                 | CPPCT19                 | UDAp-429           |         |            |
| EMPaS01A                | CPPCT22                 | UDP96-019          |         |            |
| EMPaS02B                | CPPCT23                 | UDP97-403b         |         |            |
| EMPaS10A                | CPPCT26                 | UDP98-406          |         |            |
| EMPaS11A                | CPPCT6                  |                    |         |            |
| EPPB4216-PR51           | CPSCT021/AY426206       |                    |         |            |
| EPPCU0961               | CPSCT029/AY426214       |                    |         |            |
| EPPCU3090               | CPSCT034/AY426219       |                    |         |            |
| EPPCU9168               | CPSCT038+/AY426223      |                    |         |            |
| MA007a                  | EPDCU3083               |                    |         |            |
| MA039a                  | EPDCU5060               |                    |         |            |
| MA040a                  | EPDCU5183               |                    |         |            |
| MA061a                  | EPPCU0888               |                    |         |            |
| PceGA34                 | EPPCU1589               |                    |         |            |
| PceGA59                 | EPPCU2828               |                    |         |            |
| pchcms4                 | EPPCU3664               |                    |         |            |
| pchcms5                 | EPPCU3990               |                    |         |            |
| PMS3                    | EPPCU8702               |                    |         |            |
| PMS49                   | MA005c                  |                    |         |            |
| PMS67                   | MA056a                  |                    |         |            |
| UCD-CH12                | MA069a                  |                    |         |            |
| UCD-CH31                | MD201a                  |                    |         |            |
| UDAp-426                | PMS30                   |                    |         |            |
| UDP96-001b              | PMS40                   |                    |         |            |
| UDP96-005               | UDAp-427                |                    |         |            |
| UDP98-021               | UDAp-407                |                    |         |            |
| UDP98-022               | UDAp-418                |                    |         |            |
| UDP98-410               | UDAp-461                |                    |         |            |
|                        |                        |                    |         |            |