# A New System for Strawberry Cultivar Identification Developed at Foundation Plant Services (FPS), University of California, Davis, Using Simple Sequence Repeat (SSR) Primers

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**Abstract. Early and correct cultivar identification of strawberry (*Fragaria ×ananassa* Duch.), a clonally propagated crop with an octaploid genome, is critical to the industry. Sixteen previously published primer pairs that flanked simple sequence repeats (SSRs) in the strawberry cultivar Earliglow were examined for their ability to distinguish commonly grown strawberry cultivars. Six primer pairs both amplified consistently and produced variable fragment sizes. One primer pair, ARSFL-09, generated unique profiles for 29 of 45 accessions. The remaining 16 fell into eight profile groups of two cultivars each, which were easily resolved with any one of three other primer pairs. Although the octaploid nature of strawberry complicates interpretation, this system can reliably identify the most commonly grown strawberry cultivars at an early developmental stage.**

Strawberries are California's seventh most valuable crop, with direct agricultural sales of more than $1.2 billion in 2004 (CDFA, 2006). More than 60% of the state's strawberry acreage is planted with cultivars developed by the strawberry breeding program at the Department of Plant Sciences, UC Davis (CSC, 2006). Foundation Plant Services (FPS) is the UC service department which produces and distributes virus-tested propagation stock plants of UC-patented strawberry cultivars licensed by the UC Office of Technology Transfer. Cultivated strawberry (*Fragaria ×ananassa* Duch.) plants are vegetatively propagated. Maintaining correct cultivar identity during this process is critical, a task made difficult because propagation stock is distributed prior to development of distinguishing morphological characteristics.

Many biochemical and molecular marker systems have been used for strawberry cultivar identification, including isozymes (Bell and Simpson, 1994), Randomly amplified polymorphic DNA (RAPD) markers (Degani et al., 1998; Garcia et al., 2002; Hancock et al., 1994), Amplified Fragment Length Polymorphism (AFLP) markers (Miroslaw et al., 2002), and Inter Simple Sequence Repeat (ISSR) markers (Arnau et al., 2002). Each of these systems has its own technical limitations; all have known problems with reproducibility of data among labs.

Simple sequence repeats (SSRs) are abundant and scattered throughout the eukaryotic genome (Hamada et al., 1982). They have been widely used for cultivar identification in grape, walnut, and other clonally propagated crops (Dangl et al., 2001, 2005), but SSR markers take considerable time and expertise to develop (Lewers et al., 2005). Application of this technology to strawberry is particularly difficult because strawberries are octaploid, with eight copies of each gene, and because inbreeding can lead to cultivars with multiple copies of the same allele at a single locus. Since SSR technology can detect only the presence or absence of an allele, but not copy number, there are often several genetic combinations that could lead to a particular profile.

Here we describe an SSR marker system that can distinguish closely related, UC-patented strawberry cultivars that are widely planted in California. This system uses published markers to reduce development time, and scores the amplified fragments as dominant markers. Because primer binding is highly specific, this system is both highly accurate and reproducible by other laboratories.

## Materials and Methods

**Plant material and DNA extractions.** Initial young leaves were collected and stored frozen from a set of 23 accessions of cultivated strawberry; these included cultivars and selections from the UC Davis breeding program and one diverse cultivar (Table 1). The frozen leaves were ground and DNA extracted using the DNeasy 96 Plant Kit (Qiagen Inc., Valencia, CA) according to the manufacture's protocol. Leaves from a second set of accessions (Table 1) were dried rapidly using Drierite (W.A. Hammond Drierite Co., Xenia, OH) prior to DNA extraction. Subsequently, DNA has been successfully extracted from various other tissues: root, stem, calyx, and fruit, using several standard protocols. A total of 45 accessions were genotyped with six selected primer pairs using the optimized PCR protocol. Multiple plants were tested for most accessions.

**Amplification conditions.** Sixteen primer pairs (Table 2) for PCR amplification of SSR containing loci cloned from the strawberry cultivar 'Earliglow' (Lewers et al., 2005; Nourse et al., 2002) were screened with the initial set of 23 strawberry accessions using a range of PCR reaction mixes and thermal cycle profiles. The optimal PCR reaction mix was approximately 6.25 ng·µL$^{-1}$ genomic DNA, 2 mM $MgCl_2$, 0.8 mM of each dNTP [dNTP mix, Applied Biosystems Inc. (ABI), Foster City, CA], 0.025 units/µL AmpliTaq Gold (ABI), 1× Gold Buffer (ABI), and 0.25 pmol·µL$^{-1}$ of each primer in a total volume of 20 uL. One primer of each pair was labeled with a fluorescent dye. For the

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Table 1. Strawberry (*Fragaria ×ananassa*) cultivars and selections used in this study.

| Accession[z] | Accession |
|---|---|
| Aromas | Aiko |
| Albiony | Anaheim |
| Camarosa | Aptos |
| Camino Real | Brighton |
| Chandler | Capitola |
| Diamante | Carlsbad |
| Douglas | Cruz |
| Gaviota | Cuesta |
| Irvine | Fern |
| Kent | Fresno |
| Oso Grande | Hecker |
| Pacific | Laguna |
| Pajaro | Mrak |
| Parker | Muir |
| Red Chief | Palomar |
| Seascape | Santana |
| Selva | Soquel |
| Ventana | Sunset |
| Breeder's selection A | Tioga |
| Breeder's selection B | Toro |
| Breeder's selection C | Totem |
| Breeder's selection D | Yolo |
| Breeder's selection E | |

[z]Twenty-three accessions used as the primary screen.

Table 2. Sixteen primer pairs tested.[z]

| | | |
|---|---|---|
| ARSFL-01 | ARSFL-10 | ARSFL-17 |
| ARSFL-02 | ARSFL-11 | ARSFL-19 |
| ARSFL-03 | ARSFL-12 | ARSFL-22 |
| ARSFL-04 | ARSFL-14 | ARSFL-27 |
| ARSFL-07 | ARSFL-15 | ARSFL-29 |
| ARSFL-09 | | |

[z]Primer sequences now published in K.S. Lewers, S.M.N. Styan, and S.C. Hokanson. 2005. Strawberry GenBank-derived and genomic simple sequence repeat (SSR) markers and their utility with strawberry, blackberry, and red and black raspberry. J. Amer. Soc. Hort. Sci. 130(1):102–115.

initial screen, reverse primers were labeled with the fluorescent dye FAM. The internal size standard was 400HD-ROX (ABI). Following screening, selected primer pairs were labeled with the fluorescent dyes HEX and NED, allowing analysis of fragments from three primer pairs per injection. The optimal thermal cycle profile consisted of 5 min at 94 °C, 40 cycles of 30 s at 94 °C, 45 s at 52 °C, and 1 min at 72 °C, followed by 20 min at 72 °C.

**Fragment separation and sizing.** Amplified fragments were separated by capillary electrophoresis on an ABI Prism 3730 Genetic Analyzer using standard run parameters with POP4 as the matrix (ABI). Depending on the concentration of amplified product, injection samples were prepared by mixing 0.5 to 0.8 uL amplified product from separate reactions with 0.5 uL size standard and 10 uL formamide. Fragment size, peak binning, and label editing were performed using several versions of GeneScan and GenoTyper Software (ABI).

## Results and Discussion

Twenty-three strawberry accessions were selected for initial examination (Table 1). These included both selections from the UC Davis breeding program and an unrelated cultivar. Fragments of DNA were amplified using 14 of 16 tested primer pairs (Table 2). The primer pairs ARSFL-03 and ARSFL-29 failed to amplify fragments under any PCR reaction conditions tested, including various thermal cycle profiles and concentrations of MgCl, primers, and template DNA. The remaining 14 primer pairs amplified peaks under different PCR conditions. (Since amplified DNA fragments separated by capillary electrophorisis are represented as peaks in GeneScan and GenoTyper software, we use the term "peak" here in the same way the term "band" is used in gel-based systems.) Peaks were inconsistently amplified by some primer pairs, while other primer pairs produced identical peak patterns for all 23 initial accessions.

An efficient system for routine identification of strawberry cultivars requires a uniform protocol that produces consistent peaks with patterns that are highly variable among the population to be tested. Using one optimal PCR condition, six primer pairs amplified reproducible peaks. Peaks that amplified consistently and were variable among the study set were scored as present or absent (Table 3). Peak profiles for each primer pair were represented in several formats (Table 4). These six primers were used to genotype the full set of accessions.

The complexity of the peak patterns and their ability to distinguish among strawberry accessions varied among these six primer pairs (Table 5). The primer pair with the most diverse peaks, ARSFL-09, generated unique profiles for 29 of the 45 accessions. The remaining 16 fell into eight profile groups of two cultivars each. These pairs were easily resolved with any one of three other primer pairs. The two primer pairs ARSFL-09 and ARSFL-10 alone are most likely able to uniquely characterize all strawberry cultivars.

The peaks observed in this study had "+A additions" and "-2 stutter," which are typical minor peaks associated with PCR amplification of SSR-containing loci. The primer pairs tested in this study are homologous to flanking regions on opposite sides of known simple sequences repeats, and are between 16 and 24 base pairs in length. The degree of primer specificity and the peak structure demonstrate that each primer pair is amplifying a specific locus and that the complex peak patterns result from amplification of multiple copies of the locus within the octoploid genome of cultivated strawberry. Although they result from highly specific amplification, the peaks do not behave as Mendalian

Table 3. Polymorphic markers "peaks" scored in base pairs for six primer pairs selected for routine cultivar identification.

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ARSFL-09 | 199 | 201 | 202 | 203 | 205 | 209 | 211 | 213 | 215[z] | 216 | 218 | 219 | 221 | 224 |
| ARSFL-10 | 210 | 219 | 221 | 225 | 229 | 234 | 236 | 240 | 243 | 246 | 264[z] | | | |
| ARSFL-11 | 253 | 259 | 261 | 263 | 267 | 269[z] | 273 | | | | | | | |
| ARSFL-12 | 256[z] | 262 | 268 | | | | | | | | | | | |
| ARSFL-15 | 172 | 180 | 184 | 186[z] | 188 | 214 | 242 | | | | | | | |
| ARSFL-17 | 200 | 202[z] | 204[z] | 210 | 212 | 219 | 231 | | | | | | | |

[z]High frequency peaks.

Table 4. ARSFL-15 peak profiles "DNA fingerprints" for selected strawberry cultivars.

| Reference cultivar | Polymorphic peaks[z] scored in base pairs | | | Polymorphic peaks scored present or absent | | | | | | | Profile code |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 172 | 180 | 184 | 186 | 188 | 214 | 242 | |
| Aiko | 186 | 188 | 214 | | | | 1 | 1 | 1 | | A |
| Carsbad | 186 | 188 | 214 | | | | 1 | 1 | 1 | | A |
| Cuesta | 186 | 188 | 214 | | | | 1 | 1 | 1 | | A |
| Cruz | 188 | 214 | | | | | | 1 | 1 | | B |
| Pajaro | 188 | 214 | | | | | | 1 | 1 | | B |
| Soquel | 188 | 214 | | | | | | 1 | 1 | | B |
| Chandler | 186 | 214 | | | | | 1 | | 1 | | C |
| Hecker | 186 | 214 | | | | | 1 | | 1 | | C |
| Mrak | 186 | 214 | | | | | 1 | | 1 | | C |
| Muir | 186 | 188 | | | | | 1 | 1 | | | D |
| Yolo | 186 | 188 | | | | | 1 | 1 | | | D |
| Anaheim | 186 | | | | | | 1 | | | | E |
| Laguna | 186 | | | | | | 1 | | | | E |
| Cultivar A | 188 | | | | | | | 1 | | | F |
| Cultivar B | 188 | | | | | | | 1 | | | F |
| Capitola | 180 | 186 | | | 1 | | 1 | | | | G |
| Sunset | 180 | 186 | | | 1 | | 1 | | | | G |
| Aptos | 186 | 188 | 242 | | | | 1 | 1 | | 1 | H[y] |
| Fresno | 172 | 186 | 214 | 1 | | | 1 | | 1 | | I[y] |
| Red Chief | 184 | | | | | 1 | | | | | J[y] |
| Santana | 180 | 186 | 214 | | 1 | | 1 | | 1 | | K[y] |
| Totem | 172 | | | 1 | | | | | | | L[y] |

[z]These peaks are polymorphic among the 45 strawberry accessions. Nearly monomorphic peaks are present at 178 and 230 base pairs.
[y]These cultivars are uniquely identified with only this primer pair.

Table 5. Six primer pairs selected for routine cultivar identification.

| | Primer Pairs | | | | | |
|---|---|---|---|---|---|---|
| | ARSFL-09 | ARSFL-10 | ARSFL-11 | ARSFL-12 | ARSFL-15 | ARSFL-17 |
| Peaks scored per primer pair[z] | 14 | 11 | 7 | 3 | 7 | 7 |
| Maximum number of peaks scored in an accession | 6 | 5 | 4 | 2 | 3 | 4 |
| Unique peak profiles | 37 | 29 | 13 | 5 | 12 | 14 |
| Accessions uniquely identified | 29 | 22 | 3 | 1 | 5 | 6 |

[z]Only peaks that amplified consistantly and were variable among the accessions are included.

alleles, because copy number cannot accurately be determined with this system. Thus, peaks were treated as dominant markers, and we continue to use the term primer pair rather than locus or marker.

## Conclusion

Six of 16 previously published primer pairs from strawberry both amplified consistently and produced variable peak sizes over our study set of 45 commonly grown strawberry cultivars and breeder's selections. One primer pair, ARSFL-09, generated unique profiles for 29 of the 45 accessions. The remaining 16 fell into eight profile groups of two cultivars each, which were easily resolved with any one of three other primer pairs. Although the octaploid nature of strawberry complicates interpretation, this system can reliably identify strawberry cultivars at an early developmental stage.

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