Strawberry GenBank-derived and Genomic Simple Sequence Repeat (SSR) Markers and Their Utility with Strawberry, Blackberry, and Red and Black Raspberry

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ABSTRACT. Although simple sequence repeat (SSR) markers have been developed for species in the closely related genera Fragaria L. (strawberry) and Rubus L. (raspberry and blackberry), the number of SSRs available is insufficient for genetic mapping. Our objective was to use and compare multiple approaches for developing additional SSRs for Fragaria and Rubus. The approaches included: the development of SSRs from GenBank sequences from species of varied relatedness to Fragaria and Rubus and identified with two different data-mining methods (BLAST and SSRIT); the evaluation of some previously published SSRs designed from related species; and the development of SSRs from a genomic library made from F. ×ananassa Duschene ex Rozier ‘Earliglow’. When an SSR was developed from a known gene sequence, the location of the repeat in the gene was determined to evaluate the effect on amplification and polymorphism detection. Cross-generic amplification between closely related Fragaria and Rubus as well as transference from species of varied relatedness to Fragaria and Rubus also was evaluated and indicated limited transference within the subfamily Rosoideae. However, development of SSRs for Fragaria and Rubus from Rosa L. (rose) and Rosaceae genera outside Rosoideae was not efficient enough to be practical for new map development. SRRT was superior to BLAST for identifying GenBank sequences containing repeats. SSRs developed from repeats found in either the 5´UTR (80% polymorphic) or 3´UTR (85% polymorphic) were most likely to detect polymorphisms, compared with those developed from coding regions (30%). SSRs developed from the genomic library were only slightly superior to GenBank-derived SSRs in their ability to detect polymorphisms.

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compare multiple approaches for developing additional SSRs for *Fragaria* and *Rubus*. The approaches included the development of new SSRs from GenBank sequences from species of varied relatedness to *Fragaria* and *Rubus* and identified with two different data-mining methods (BLAST and SSRIT); the evaluation of some previously published SSRs designed from related species; and the development of new SSRs from a genomic library made from *F. xananassa*. When an SSR was developed from a known gene sequence, the location of the repeat in the gene was determined to evaluate the effect on amplification and polymorphism detection. Cross-generic amplification between closely related *Fragaria* and *Rubus* as well as transference from species of varied relatedness to *Fragaria* and *Rubus* also was evaluated.

**Materials and Methods**

**DETERMINING FUNCTIONAL TAXONOMIC DISTANCE FOR SSR DEVELOPMENT.** An initial study was conducted to determine how taxonomically distant the source of the repeat sequence could be and still supply primers that would amplify a product from the two target species. We tested six (CT)$_n$-containing primer pairs from each of nine genera with varied relatedness to *Fragaria* and *Rubus*. We searched GenBank using the National Center for Biotechnology Information’s (NCBI) BLAST program (Altschul et al., 1990; National Center for Biotechnology Information, 2004) for genomic and EST sequences containing CT repeats in nine genera, using the sequence (CT)$_n$ as the query sequence. A total of 54 sequences containing (CT)$_n$ repeats were selected for primer design. The taxonomically farthest genus from which primers were designed was within Rosidae, which includes the model species, *Arabidopsis thaliana* (L.) Heynh.; six repeat-containing *A. thaliana* sequences were selected for primer design. From the next taxonomic level closer to *Fragaria* and *Rubus*, the eurasids I, 12 primers were designed from the Fabaceae Lindl., nom. cons. family [six from *Glycine max* (L.) Merr. (soybean); and six from *Medicago truncatula* Gaertn. (a model legume)], and six from the Malvaceae family [Gossypium L. (cotton)]. Within the Rosaceae family, six primer pairs were designed from *Prunus persica* representing the subfamily Prunoideae, and 12 primer pairs were designed from species representing the subfamily Maloideae (six from *Pyrus communis* L. and six from *Malus ×domestica* Borkh.). In the subfamily Rosoideae, to which the two target genera, *Fragaria* and *Rubus*, belong, six primer pairs were obtained from *F. xananassa* and six from *Rosa* (rose). Primers were developed with the on-line version of Primer3 software (Whitehead Institute for Biomedical Research, 2004) using the default settings (Rozen and Skaletsky, 2000). The recommended primers were synthesized without modification by Operon Technologies (Qiagen, Valencia, Calif.). In addition, several published SSR primers were tested and included 21 primer pairs designed from *Prunus persica* and reported to amplify a product from strawberry (Dirlewanger et al., 2002); eight primer pairs developed from *Rubus alceifolius* (Amsellem et al., 2001); four SSR primer pairs designed from *F. virginiana* genomic sequences (Ashley et al., 2003); and 10 designed from *F. vesca* (diploid) genomic sequences (James et al., 2003).

**DEVELOPMENT OF ADDITIONAL SSRs FROM FRAGARIA SEQUENCES IN GENBANK.** Two methods were used to identify GenBank *Fragaria* sequences containing repeats. The first was the direct BLAST search method used above with (CT)-containing SSRs to determine the taxonomic distance for SSR transference to *Fragaria* and *Rubus*. Genomic and EST sequences of *Fragaria* GenBank sequences were searched using all possible (dinucleotide)$_n$ and (trinucleotide)$_n$ repeats as a query sequence. The second method of finding GenBank sequences containing repeats used the Perl script SSRIT (simple sequence repeat identification tool) (Gramene, 2004; Temnykh et al., 2001). A total of 306 genomic and EST *Fragaria* sequences present in the nucleotide database of the NCBI were screened for repeat sequences. The maximum motif length group selected was pentamer, and the minimum number of tandem repeats was set at five (and then also later at four to identify additional repeat-containing sequences).

The location of the simple sequence repeat in the gene was determined to evaluate the effect on amplification and polymorphism detection. If available, the genomic as well as the coding sequence of the repeat-containing GenBank sequences were aligned using the CLUSTALW algorithm (Institute for Chemical Research, 2000; Thompson et al., 1994), enabling the identification of the repeat location with respect to the gene. Alternatively, the repeat-containing sequence was compared against known gene sequences archived in GenBank using the BLASTN algorithm. For each sequence, the location of the repeat region [5¢ untranslated region (5¢ UTR), intron, exon, or 3¢ untranslated region (3¢ UTR)] was determined. Whenever possible, primers were designed in the coding regions of the gene. After eliminating duplicated sequences and sequences that contained repeats at their ends, primer pairs were designed from 25 sequences using Primer3 (Table 1).

**SSR DEVELOPMENT FROM A GENOMIC LIBRARY.** *Fragaria xananassa* ‘Earliglow’ genomic DNA was digested with restriction enzymes Sma I, Msc I, and Rsa I. The resulting DNA fragments were phosphorylated and electrophoretically separated through a 0.8% agarose, 1×TBE gel. The DNA fragments between 400–800 base pairs (bp) were extracted and purified from the gel according to the QIAquick Gel Extraction Kit protocol (Qiagen). Purified DNA fragments were ligated into the *Sma I* site of the pUC 19 vector with T4 ligase (New England Biolabs, Beverly, Mass.), and transformed into Epicurian Coli Ultracompetent Cells (Stratagene, La Jolla, Calif.). Transformed cells were grown on LB-agar plates containing 50 mg·mL–1 of ampicillin, 40 mg·mL–1 of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and 40 mg·mL–1 IPTG (isopropyl-1-thio-β-D-galactopyranoside). White colonies were transferred to Optitran BA-S85 membranes (Schleicher & Schuell, Keene, N.H.), and hybridized with 32P-labeled (CT)$_n$ probe. After three rounds of screening, single bacterial colonies were grown and selected for plasmid DNA isolation using the QIAprep Miniprep Kit (Qiagen).

Plasmid DNA from the selected positive clones was diluted to 100 ng·µL–1 for sequencing reactions. Clone inserts were sequenced from both ends using M13 17-mer forward and 24-mer reverse sequencing primers (New England Biolabs) in the BigDye Terminator Cycle sequence ready reaction mixture (Applied Biosystems, Foster City, Calif.) according to the supplied protocol. The reaction products were analyzed with an ABI 310 Automated DNA Sequencer (Applied Biosystems). Repeat-containing sequences were compared against known gene sequences contained in GenBank using the BLASTN and BLASTX algorithms.

Oligo Lite, Version 6 (Molecular Biology Insights, Cascade, Colo.) was used for primer design. Primers were chosen under the highest stringency conditions possible with criterion including primer efficiency (PE) values >400, duplex values >4, and a melting temperature difference of ≤20 °C between primer pairs. Twenty-eight primer pairs were synthesized by BioServe Biotechnologies, Ltd. (Laurel, Md.) (Table 1).


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GENOTYPES USED FOR PRIMER TESTING IN FRAGARIA AND RUBUS.
SSR primers were tested in PCR amplification with genomic DNA from 31 genotypes (Table 2): 12 F. xananassa (octoploid) genotypes (cultivated octoploid strawberry), five F. chiloensis (L.) Mill. genotypes (octoploid maternal progenitor species of cultivated strawberry), three F. virginiana genotypes (octoploid paternal progenitor species of cultivated strawberry), one F. virginiana × F. xananassa hybrid (octoploid), four F. vesca (diploid) genotypes, one F. iinumae Makino (diploid), and six Rubus genotypes (four tetraploid blackberry, one diploid red raspberry, and one diploid black raspberry). Many of these genotypes are parents of existing mapping populations and were selected so that we could obtain polymorphism data for multiple mapping populations (Tables 2 and 3). Plants were grown at multiple locations: the USDA–ARS National Clonal Germplasm Repository at Corvallis, Ore.; the USDA–ARS Beltsville Agricultural Research facility at Beltsville, Md.; the Univ. of Arkansas station near Clarksville, Ark.; the USDA–ARS Sunnyvale, Calif.), and diluted with 1x TE buffer. At Beltsville, DNA was extracted using Qiagen’s DNeasy Plant Maxi Kit or DNeasy Plant Mini Kit. DNA was quantified using the Spectraxmax 190 spectrophotometer (Molecular Devices, Sunnyvale, Calif.), and diluted with 1x TE buffer to 30 ng·µL–1. Dilutions to 0.03 ng·µL–1 were made with ddH2O.

PCR CONDITIONS. At Corvallis, PCR amplifications were carried out in 10 µL total volume of 1x Biolase reaction buffer, 2 mM MgCl2, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.3 µM each of forward and reverse primers, 0.25 U of Biolase Taq DNA polymerase (Bioline USA, Randolph, Mass.), and 2.5 ng of template DNA. DNA was amplified for 35 cycles in an Eppendorf Gradient thermocycler (Brinkmann Instruments, Westbury, N.Y.) or an MJ Research Tetrad thermocycler (MJ Research, Waltham, Mass.) programmed for a 40-s denaturation step at 94 °C, a 40-s annealing step at the optimum annealing temperature, and a 40-s extension step at 72 °C. The optimum annealing temperature

Table 1. Primer pairs tested in polymerase chain reactions (PCRs) with template DNA from several Fragaria and Rubus genotypes. The primer pairs were either published previously or developed from nucleotide sequences containing simple sequence repeats (SSRs). The SSR-containing sequences were identified from among Fragaria or Rubus sequences archived in the GenBank database or from a genomic library made from F. xananassa ‘Earliglow’ DNA. The source sequence GenBank accession number, clone number, and/or reference (if published) for each primer pair are given. Location of the SSR with respect to the gene is indicated when known. If a primer pair amplified a product in PCR reactions with any tested genotype in the listed species, a “+” sign is indicated. If the primer pair also detected polymorphisms among the tested genotypes in the listed species, a “+/+” sign is indicated. If the primer pair did not amplify a product from any of the genotypes tested in the listed species, a “−” sign is indicated. If the primer pair was not tested on any of the genotypes in the species, no sign is given.

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<td>ARSFL_18</td>
<td>14F5, 'Earliglow', NS</td>
<td>F: gcgcgaacctcatagacact tgtcgc R: gcgcgctccctgatacaaatc</td>
<td>52</td>
<td>291</td>
<td>(ct)\textsubscript{23-1}</td>
<td>- - - - -</td>
</tr>
<tr>
<td>ARSFL_19</td>
<td>14G5, 'Earliglow', NS</td>
<td>F: gcgcgaacctcatagacact tgtcgc R: gcgcgctccctgatacaaatc</td>
<td>52</td>
<td>251</td>
<td>(ga)\textsubscript{12}</td>
<td>+/+ + + - - +</td>
</tr>
<tr>
<td>ARSFL_20</td>
<td>15A4, 'Earliglow', NS</td>
<td>F: gcgcgaacctcatagacact tgtcgc R: gcgcgctccctgatacaaatc</td>
<td>52</td>
<td>190</td>
<td>(ct)\textsubscript{19-1}</td>
<td>- - - - - -</td>
</tr>
<tr>
<td>ARSFL_22</td>
<td>15E7, 'Earliglow', NS</td>
<td>F: gcgcgaacctcatagacact tgtcgc R: gcgcgctccctgatacaaatc</td>
<td>52</td>
<td>158</td>
<td>(ga)\textsubscript{11}</td>
<td>+/- +/+ +/+ +/+ +/+</td>
</tr>
<tr>
<td>ARSFL_23</td>
<td>16D6, 'Earliglow', NS</td>
<td>F: gcgcgaacctcatagacact tgtcgc R: gcgcgctccctgatacaaatc</td>
<td>52</td>
<td>237</td>
<td>(ga)\textsubscript{13}</td>
<td>- + + +/+ +/+</td>
</tr>
<tr>
<td>ARSFL_24</td>
<td>16F8, 'Earliglow', NS</td>
<td>F: gcgcgaacctcatagacact tgtcgc R: gcgcgctccctgatacaaatc</td>
<td>52</td>
<td>195</td>
<td>(ct)\textsubscript{20}</td>
<td>+ - + +/+ -</td>
</tr>
<tr>
<td>ARSFL_26</td>
<td>17A1, 'Earliglow', NS</td>
<td>F: gcgcgaacctcatagacact tgtcgc R: gcgcgctccctgatacaaatc</td>
<td>52</td>
<td>282</td>
<td>(ga)\textsubscript{12}</td>
<td>+ + + +/+ -</td>
</tr>
<tr>
<td>ARSFL_27</td>
<td>17C4, 'Earliglow', NS</td>
<td>F: gcgcgaacctcatagacact tgtcgc R: gcgcgctccctgatacaaatc</td>
<td>52</td>
<td>164</td>
<td>(ct)\textsubscript{46-1}</td>
<td>+ + + +/+ -</td>
</tr>
</tbody>
</table>

Table 1 continued next page
### Table 1. Continued.

<table>
<thead>
<tr>
<th>Marker</th>
<th>GenBank or library accession, source genome, sequence similarity</th>
<th>Primer sequences 5' to 3', or source reference if published</th>
<th>Tm °C</th>
<th>Size (bp)</th>
<th>Repeat</th>
<th>Location</th>
<th>F. × ananassa (diploid)</th>
<th>F. × ananassa (octoploid)</th>
<th>F. × virgíniana (octoploid)</th>
<th>Blackberry (tetraploid)</th>
<th>Raspberry (diploid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARSFL_28</td>
<td>'Earliglow', NS</td>
<td>F:gcgcggctgtttttctctttgt, R:gcgcggctttctactgggaacaaag</td>
<td>52</td>
<td>287</td>
<td>(ga)18</td>
<td>+ + + +/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ARSFL_29</td>
<td>'Earliglow', NS</td>
<td>F:gcgcggctgtttttctctttgt, R:gcgcggctttctactgggaacaaag</td>
<td>52</td>
<td>284</td>
<td>(ga)13,1</td>
<td>+ - - +/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ARSFL_92</td>
<td>'Earliglow', NS</td>
<td>F:tcgggtgaanatatcaaggg, R:gggggttcgctgatcct</td>
<td>59</td>
<td>179</td>
<td>(ct)4(gp)13,1</td>
<td>+ + + +</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ARSFL_96</td>
<td>'Earliglow', NS</td>
<td>F:gcggggtgctgctgggt, R:ccgaggggaagcgagcatga</td>
<td>59</td>
<td>208</td>
<td>(ct)24</td>
<td>+ + + +/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*ARSFL primer pairs were designed in Beltsville, Md., and FAC primer pairs were developed in Corvallis, Ore.

**Similarity of the source sequence to other sequences archived in the GenBank database are provided if possible; if the similarity is not available, “NA” is indicated, and, if no similarity was found, “NS” is indicated.

**Sometimes the cultivar name was provided for F. × ananassa-derived sequences; if a cultivar name is provided, it is F. × ananassa unless otherwise stated.

**The expected PCR product size in base pairs (bp) is based on the sequence from which each primer pair is derived.

**The simple sequence motifs identified in the source sequences are indicated as the simple sequence repeats, enclosed in parentheses, and the number of repeats found in the source sequence in subscript. For example, (ct)4 is interpreted as “ctctctct”. When the subscript number is indicated as, for example, n-1, this means the repeat was not perfect and either a base pair was missing or a base pair substitution was present in the repeat, compared with a perfect repeat sequence.

### Table 2. Fragaria (strawberry) and Rubus (raspberry and blackberry) genotypes were used to evaluate published and newly developed simple sequence repeat (SSR) primers in polymerase chain reactions (PCRs).

<table>
<thead>
<tr>
<th>Accession or plant patent no</th>
<th>Plant species</th>
<th>Cultivar or other designator</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI 615055</td>
<td>F. × ananassa</td>
<td>CFRA 1008</td>
<td>Another diploid species</td>
</tr>
<tr>
<td>PI 551057</td>
<td>F. vesca</td>
<td>‘Baron Solemacher’</td>
<td>Diploid strawberry mapping population</td>
</tr>
<tr>
<td>None</td>
<td>F. vesca</td>
<td>WC6</td>
<td>Diploid strawberry mapping population</td>
</tr>
<tr>
<td>PI 9000</td>
<td>F. vesca</td>
<td>UC5</td>
<td>Diploid virus indicator</td>
</tr>
<tr>
<td>PI 551646</td>
<td>F. vesca subsp. bracteata</td>
<td>CFRA 389</td>
<td>Diploid core accession, day-neutral</td>
</tr>
<tr>
<td>PI 551908</td>
<td>F. vesca subsp. vesca</td>
<td>‘Snow King’</td>
<td>White-fruited diploid strawberry</td>
</tr>
<tr>
<td>PI 614290</td>
<td>F. chiloensis</td>
<td>‘Scott’s Creek’</td>
<td>Large-fruited, super core octoploid</td>
</tr>
<tr>
<td>PI 614289</td>
<td>F. chiloensis</td>
<td>HM1</td>
<td>Highly productive, super core octoploid</td>
</tr>
<tr>
<td>PI 552038</td>
<td>F. chiloensis</td>
<td>Island of Lenouy</td>
<td>White-fruited octoploid</td>
</tr>
<tr>
<td>PI 551736</td>
<td>F. chiloensis</td>
<td>CFRA 372</td>
<td>Super core octoploid</td>
</tr>
<tr>
<td>CFRA 1806</td>
<td>F. virginiana</td>
<td>US 4808</td>
<td>Octoploid resistant to Xanthomonas fragariae</td>
</tr>
<tr>
<td>CFRA 1807</td>
<td>F. virginiana × (F. × ananassa)</td>
<td>US 4809</td>
<td>Octoploid resistant to Xanthomonas fragariae</td>
</tr>
<tr>
<td>PI 615651</td>
<td>F. virginiana subsp. grayana</td>
<td>CFRA 1170</td>
<td>Octoploid core accession</td>
</tr>
<tr>
<td>PI 552250</td>
<td>F. virginiana subsp. virginiana</td>
<td>WC 26</td>
<td>Octoploid day-neutral</td>
</tr>
<tr>
<td>PP 9,866</td>
<td>F. × ananassa</td>
<td>‘Rosa Linda’</td>
<td>Florida-bred octoploid cultivar</td>
</tr>
<tr>
<td>PP 5,266</td>
<td>F. × ananassa</td>
<td>‘Selva’</td>
<td>Day-neutral octoploid parent in mapping population with ‘Delmarvel’</td>
</tr>
<tr>
<td>PI 615689</td>
<td>F. × ananassa</td>
<td>‘Delmarvel’</td>
<td>Phytophthora Fragariae-resistant octoploid parent in mapping population with ‘Selva’</td>
</tr>
</tbody>
</table>

*Table 2 continued next page*
Table 2. Continued.

<table>
<thead>
<tr>
<th>Accession or plant patent no</th>
<th>Plant species</th>
<th>Cultivar or other designer</th>
<th>Location tested</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td><em>F. xananassa</em></td>
<td>'Pelican'</td>
<td>B</td>
<td><em>Colletotrichum</em> species crown and fruit rot-resistant octoploid parent in mapping population with 'Chandler'</td>
</tr>
<tr>
<td>PP 5,262</td>
<td><em>F. xananassa</em></td>
<td>'Chandler'</td>
<td>B</td>
<td>Widely adapted octoploid parent in mapping population with 'Pelican'</td>
</tr>
<tr>
<td>PP 8,729</td>
<td><em>F. xananassa</em></td>
<td>'Sweet Charlie'</td>
<td>B</td>
<td>Susceptible Florida-bred octoploid parent cultivar in mapping populations with US 4808 and US 4809</td>
</tr>
<tr>
<td>PI 551394</td>
<td><em>F. xananassa</em></td>
<td>'Earlglow'</td>
<td>B,C</td>
<td>Octoploid genotype from which a genomic library for SSR development was derived</td>
</tr>
<tr>
<td>PI 551398</td>
<td><em>F. xananassa</em></td>
<td>'Titan'</td>
<td>C</td>
<td>Octoploid cultivar</td>
</tr>
<tr>
<td>PI 551416</td>
<td><em>F. xananassa</em></td>
<td>'Scott'</td>
<td>C</td>
<td>Red stele-resistant octoploid cultivar</td>
</tr>
<tr>
<td>PI 551406</td>
<td><em>F. xananassa</em></td>
<td>'Allstar'</td>
<td>C</td>
<td>Widely adapted mid-Atlantic octoploid cultivar</td>
</tr>
<tr>
<td>PI 551490</td>
<td><em>F. xananassa</em></td>
<td>'Hecker'</td>
<td>C</td>
<td>First day-neutral octoploid cultivar released in U.S.</td>
</tr>
<tr>
<td>None</td>
<td><em>R. hybrid</em> (blackberry)</td>
<td>APF12</td>
<td>B</td>
<td>Primocane-fruited tetraploid blackberry in mapping population with 'Arapaho'</td>
</tr>
<tr>
<td>PP 8,510</td>
<td><em>R. hybrid</em> (blackberry)</td>
<td>'Arapaho'</td>
<td>B</td>
<td>Summer-fruited tetraploid blackberry in mapping population with APF12</td>
</tr>
<tr>
<td>PI 553276</td>
<td><em>R. hybrid</em> (blackberry)</td>
<td>'Merton'</td>
<td>B</td>
<td>Tetraploid source of thornless trait in U.S. blackberry genotypes</td>
</tr>
<tr>
<td>PI 553348</td>
<td><em>R. hybrid</em> (blackberry)</td>
<td>'Illini Hardy'</td>
<td>B</td>
<td>Tetraploid blackberry that produces secondary floral initiation buds to replace those damaged by cold</td>
</tr>
<tr>
<td>None</td>
<td><em>R. idaeus</em> (red raspberry)</td>
<td>NY322</td>
<td>B</td>
<td>Diploid red raspberry in mapping population with 'Jewel'</td>
</tr>
<tr>
<td>PI 553742</td>
<td><em>R. occidentalis</em> (black raspberry)</td>
<td>'Jewel'</td>
<td>B</td>
<td>Diploid black raspberry in mapping population with NY322</td>
</tr>
</tbody>
</table>

*PI numbers refer to accessions in the USDA–ARS germplasm collection. CFRA numbers refer to accessions in the USDA–ARS germplasm collection at Corvallis that have not yet been given a PI number. PP numbers refer to US Plant Patent numbers. Some accessions were tested at Beltsville, Md. (B), and some were tested at Corvallis, Ore. (C). |

Table 3. Simple sequence repeat (SSR) primers derived from *Fragaria* (strawberry) and *Rubus* (bramble) sequences were tested in polymerase chain reactions (PCRs) with strawberry, blackberry, and raspberry genotypes. For each group of tested genotypes, the number of primers that were tested, the number that amplified a product, and the number that detected polymorphisms are reported.

<table>
<thead>
<tr>
<th>SSR sequence source</th>
<th>Number tested</th>
<th>Number amplified</th>
<th>Number polymorphic</th>
<th>Polymorphic in octoploid &amp; diploid parents</th>
<th>Polymorphic in tetraploid parents</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fragaria</em> genomic</td>
<td>28</td>
<td>24</td>
<td>24</td>
<td>19/24</td>
<td>9/18</td>
</tr>
<tr>
<td><em>Fragaria</em> GenBank</td>
<td>34</td>
<td>26</td>
<td>15</td>
<td>10/17</td>
<td>-</td>
</tr>
<tr>
<td><em>F. vesca</em></td>
<td>10</td>
<td>8</td>
<td>8</td>
<td>7/10</td>
<td>-</td>
</tr>
<tr>
<td><em>F. virginiana</em></td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>1/1</td>
<td>-</td>
</tr>
<tr>
<td><em>Rubus alceifolius</em></td>
<td>8</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strawberry</th>
<th>Blackberry</th>
<th>Raspberry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number tested</td>
<td>Number amplified</td>
<td>Number polymorphic</td>
</tr>
<tr>
<td>23</td>
<td>6</td>
<td>3/6</td>
</tr>
<tr>
<td>19</td>
<td>6</td>
<td>1/5</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>0/1</td>
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<tr>
<td>1</td>
<td>1</td>
<td>0/1</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>1/2</td>
</tr>
</tbody>
</table>

*The octoploid strawberry mapping parents are US 4808 and 'Sweet Charlie'.
*The diploid strawberry mapping parents are 'Baron Solemacher' and WC6.
*The tetraploid blackberry mapping parents are 'Arapaho' and Univ. of Arkansas selection APF-12.
*The diploid raspberry mapping parents are Cornell Univ. red raspberry selection NY322 and black raspberry cultivar Jewel.

for a primer pair was initially determined by gradient PCR using template DNA from two cultivars of *F. xananassa* ‘Rainier’ and ‘Earliglow’ at 55 °C ± 10 °C.

At Beltsville, PCR components included 1× Qiagen PCR buffer (1.5 mM MgCl₂), 0.67 mM dNTPs (higher than usual to facilitate amplification through repeat regions), 0.5 µM each primer, and 0.4 U HotStar Taq polymerase enzyme, and template DNA at either 2 ng µL⁻¹ (standard stringency) or 0.002 ng µL⁻¹ (high stringency) in a total reaction volume of 15 µL. Reaction components were heated to 95 °C for 15 min to activate the polymerase. The heat-activation period was followed by 30 cycles of 40 s at 95 °C, 40 s at 52 °C (for primers designed from the genomic library) or 59 °C (for primers designed from GenBank sequences), and 40 s at 72 °C, followed by 10 min at 72 °C and storage at 4 °C. The thermal cycler was the MJ Research Engin Tetrad with 96-well blocks. For some reactions a PCR gradient was used from 52 °C through 62 °C using the gradient option.

**PCR Fragment Analysis.** At Corvallis, PCR products and a 100-bp-size standard (Promega, Madison, Wis.) were separated on 3% GenePure LE agarose (ISC BioExpress, Kaysville, Utah) at 100 V for 180 min. The DNA fragments were visualized after ethidium bromide staining using the GelDoc 2000 gel documentation system (BioRad, Hercules, Calif.). To detect polymorphisms, fragment sizes were estimated by Quantity One software (BioRad).

To determine if the reactions resulted in a product, at Beltsville, PCR products were diluted in water and visualized after 12 min of electrophoresis through a 2% agarose gel using Invitrogen’s E-gel 96 system. Gel images were captured in .tif files using the Alpha Imager (Alpha Innotech Corp., San Leandro, Calif.), analyzed with the E-gel 96 Editor software (Alpha Innotech Corp.).

To size the PCR products and identify polymorphisms at Beltsville, fluorescently labeled primers were ordered from Applied Biosystems and PCR was repeated. Fluorescently labeled PCR products were separated by capillary gel electrophoresis and detected using the Applied Biosystems, 3700 DNA Analyzer. The size standard used was GENESCAN 400HD [ROX]. Sizing data were analyzed using ABI Genotyper (Applied Biosystems) software.

**Results.**

**Determining functional taxonomic distance for SSR development.** GenBank sequences containing (CT), repeats were obtained from *F. xananassa*, *Rosa*, *Prunus persica*, *Malus xdomestica*, *Pyrus communis*, *Medicago truncatula*, *Glycine max*, *Gossypium*, and *Arabidopsis thaliana*, and primers designed from six of each species were initially tested to see if they would amplify products from *Fragaria*, *Rubus*, *P. persica*, or *G. max* template DNA in PCR amplifications. None of the *P. persica*, *M. xdomestica*, *M. communis*, *M. truncatula*, *G. max*, *Gossypium*, or *A. thaliana* primers amplified products from *Fragaria* or *Rubus* DNA. Of the previously published SSRs tested, the 21 primer pairs designed from *P. persica* genomic sequences and reported by Dirlewanger et al. (2002) to amplify products from *Fragaria* genomic DNA, successfully amplified a product from *P. persica* DNA but failed in our attempts with varying PCR conditions to amplify products from *Fragaria* or *Rubus* DNA. In addition, only *Prunus*-derived primers amplified products from *P. persica* DNA, and only *G. max*-derived primers amplified a product from *G. max* DNA. Therefore, transference of SSRs from distantly related genera was less successful than expected based on previous reports.

Transference of SSRs among Rosoideae genera was tested using previously published SSRs from *Fragaria* and *Rubus* species, including four primer pairs designed from *F. virginiana* genomic sequences (Ashley et al., 2003), 10 SSRs designed from genomic sequences of *F. vesca* (diploid strawberry) by James et al. (2003), and eight primer pairs developed from *R. alcefolius* (Amselem et al., 2001), as well as six new GenBank-derived *F. xananassa* primers. One of the four primer pairs designed from *F. virginiana* genomic sequences (Ashley et al., 2003) amplified both blackberry and raspberry DNA. Three of the 10 SSRs designed from *F. vesca* (diploid strawberry) genomic sequences by James et al. (2003) amplified products from blackberry, and two of those amplified products from raspberry. One of the six *F. xananassa* primers amplified a product from blackberry, and one amplified a product from raspberry. In total, 27% and 19% of these *Fragaria*-derived primer pairs amplified a product in blackberry and/or raspberry, respectively. Interestingly, of the eight primer pairs developed from *R. alcefolius* (Amselem et al., 2001), only two amplified a product from blackberry and raspberry, so that using the *R. alcefolius*-derived SSRs with blackberry and raspberry was no better than using the *Fragaria*-derived SSRs. None of the eight *R. alcefolius*-derived primer pairs amplified a product from *Fragaria* DNA. In addition, none of the *Rosa*-derived primers amplified a product from *Fragaria* or *Rubus*. To confirm this finding, 24 additional *Rosa*-derived SSR primer pairs (data not shown) were tested with *Fragaria* and *Rubus* DNA, and, again, none amplified a product. Therefore, some SSRs were transferable between some Rosoideae genera, but not others.

Transference of SSRs between *Fragaria* congeners was highly successful. The published *F. virginiana* and *F. vesca* SSRs (Ashley et al., 2003; James et al., 2003) were used in addition to six GenBank *F. xananassa*-derived primers to test for intragenic amplification. These 20 primer pairs amplified products from all the *Fragaria* templates tested, including *F. xananassa*, *F. virginiana*, *F. chiloensis*, and *F. vesca* (Tables 1 and 3). These results support the conclusion made by Ashley et al. (2003) and Sargent et al. (2003) that primers designed from one *Fragaria* species generally can be used with other *Fragaria* species.

**GenBank-derived SSRs and cross-specific amplification and polymorphism in *Fragaria*.** Because transference of SSRs between *Fragaria* congeners was so successful, we developed new strawberry SSRs using *Fragaria* sequences archived in GenBank.

Of the two methods used to identify SSR-containing *Fragaria* sequences from GenBank, SSRIT was superior to BLAST in that it detected a larger number of repeats as well as a greater diversity of repeat motifs. From the 306 *Fragaria* sequences in GenBank, SSRIT identified 42 with a minimum of five tandem repeats and 227 sequences with a minimum of 4 tandem repeats. The motifs uniquely identified by SSRIT included AAAAT (FAC-001), CTT (Fac0010, FAC-010), AAG (FAC-003, FAC-013), and GCT (FAC-016). BLAST identified 57 *Fragaria* sequences containing repeats.

All primer pairs designed from GenBank sequences using both methods amplified a product in the source species *F. xananassa* except for FAC-010. SSRs FAC-012 and FAC-014, designed from *F. vesca*, amplified PCR fragments of the expected size in *F. chiloensis*, *F. virginiana*, and *F. xananassa*. It is of interest to note that FAC-012 failed to amplify in the source species, *F. vesca*. The amplification rate using GenBank-derived SSR primers was high within the *Fragaria* genus (73% in *F. vesca*; 79% in *F. tinumae*; 88% in the octoploid *F. chiloensis* and *F. virginiana*;
and 92% in the source species F. xananassa). Likelihood of polymorphism detection seemed to increase as the number of simple sequence repeats increased, though we were not able to determine if the apparent increase was statistically significant. Of GenBank-derived SSR primers that amplified a product, 68% with six or more repeats detected polymorphisms among tested accessions, and 43% with fewer than six repeats detected polymorphisms. Between the octoploid strawberry parents (US 4808 × ‘Sweet Charlie’) of a genetic mapping population, 59% of the GenBank-derived primers detected polymorphisms.

Effect of Repeat Location on Amplification and Polymorphism Detection. Simple sequence repeats were found in all regions of genes: ten were present in 5' UTRs, eight in 3' UTRs, eleven in coding regions and three were detected in introns (Table 1). The SSRs identified from the 5' UTRs contained mostly dinucleotide repeats (7 out of 10) and 80% detected polymorphisms among the F. xananassa accessions tested. All five of the dinucleotide-containing SSRs isolated from 3' UTRs detected polymorphisms among F. xananassa, while only one out of the three trinucleotide-containing SSRs detected polymorphisms. Trinucleotide repeats were the most abundant type found in the coding regions as previously reported in other plant species (Smulders et al., 1997). Seven of the eleven SSRs identified in exons contained trinucleotide repeats. Three of these trinucleotide-containing SSRs (42%) identified in Fragaria coding sequences detected polymorphisms. The number of SSRs detected in introns was small in this study. Out of 3 intron-located SSRs, only one, FAC-008, detected polymorphisms within F. vesca, F. chiloensis, F. virginiana and F. xananassa. In spite of these trends, ARSFL_33, derived from a Fragaria sequence and having only four tandem repeats in a coding region detected polymorphisms between raspberry genotypes.

SSRs Developed from a Fragaria Genomic Library. Both the amplification success rate and the percentage of polymorphism detected by SSRs derived from the genomic library were 85%. Seventy-seven percent of SSRs derived from the genomic library, averaged across F. xananassa, F. vesca, and F. virginiana, detected polymorphisms. Of the SSRs derived from the F. xananassa genomic library, 79% detected polymorphisms between parents of an octoploid population (US 4840 × ‘Sweet Charlie’) and 50% between the parents of a diploid population (‘Baron Solemacher’ × WC6) (Davis and Yu, 1997). The SSR primers derived from the genomic library detected more polymorphisms than those derived from GenBank sequences (Table 3).

Genomic- as opposed to GenBank-derived SSRs in Fragaria. There were no noticeable differences in ability to amplify a product that could be associated with SSR derivation from the genomic library or GenBank sequences. Among SSRs derived from the genomic library, all but ARSFL_18 and ARSFL_20 amplified a product from at least one genotype, and among GenBank-derived SSRs, all but FAC-010, ARSFL_105, and ARSFL_107 amplified a product. ARSFL_19, derived from the genomic library, amplified a product from some genotypes but not its source genome F. xananassa. Similarly, GenBank-derived SSRs FAC-006b and FAC-012 did not amplify a product from their respective source genomes, F. xananassa and F. vesca, though they did amplify products from other genomes. However, SSRs developed from the genomic library rather than GenBank sequences detected more polymorphisms (Table 3).

Cross-species Amplification and Polymorphism of SSRs within Fragaria. Within Fragaria, there were no significant differences in SSR ability to amplify a product associated with SSR source species (Tables 1 and 3). SSRs developed from F. xananassa, F. virginiana, and F. vesca seemed equally capable of amplifying products from any Fragaria species and detecting polymorphisms between genotypes within species. Twenty-one of the twenty-two primer pairs obtained from F. xananassa were cross-transferable to the progenitor octoploids, F. chiloensis and F. virginiana. In the diploid species, F. iinumae and F. vesca, 19 and 17 of the 22 F. xananassa primers, respectively, amplified the expected size fragments. Furthermore, 66% of the F. xananassa-derived SSRs detected polymorphisms between parents of the octoploid strawberry genetic mapping population (US 4808 × ‘Sweet Charlie’) and 56% between parents of the diploid strawberry population (‘Baron Solemacher’ × WC6).

Cross-generic Amplification and Polymorphism of SSRs between Fragaria and Rubus. Because some SSRs were transferable between some Rosoideae genera, but not others, cross-generic amplification between Fragaria and Rubus was evaluated further using new Fragaria BLAST-identified SSRs as well as 22 F. xananassa genomic SSR primer pairs. Between 26% to 31% of the new Fragaria genomic and GenBank-derived primer pairs resulted in amplification of product from blackberry and 17% to 20% from raspberry (Table 3). In total, ten Fragaria-derived SSR primers amplified products from both blackberry and raspberry, although only two detected polymorphisms. Five SSR primers amplified products from only blackberry (three detected polymorphisms), and three SSR primers amplified products from only raspberry (two detected polymorphisms). Of SSR primers that amplified a product, the percentage that detected polymorphisms ranged from 20% to 60% with 35% average for blackberry, and 43% average for raspberry.

Discussion

Transference of SSRs among distantly related genera was unsuccessful as sequences derived from other genera did not amplify a product from the Fragaria and Rubus genotypes we tested. Although rose is in the same subfamily, Rosoideae, the thirty primer pairs we designed from rose sequences did not amplify a product from any strawberry or bramble genotype. And, although SSRs derived from peach have been reported to amplify a product from strawberry, the 21 peach-derived SSRs reported by Dirlewanger et al. (2002) to amplify products from strawberry failed in our attempts with varying PCR conditions to amplify products from Fragaria or Rubus DNA. However, these and six primers we designed from peach sequences in GenBank amplified a product from peach DNA. This is especially significant, because peach recently has been proposed as a model species for the entire Rosaceae family. Our findings indicate that peach SSRs will not be as useful as initially hoped for Fragaria and Rubus species and support the findings of Peakall et al. (1998) and Decroocq et al. (2003) on limited cross-species transferability in the Rosaceae family. Decroocq and co-workers (2003) found that optimal utility of Prunus armeniaca L. (apricot) EST SSR markers was obtained for closely related species belonging to the same subgenus Prunophora, and that the threshold distance after which no amplification can be expected is short in the Rosaceae family. It is likely that a small percentage of primers designed from Prunus and other Rosaceae genera will reliably amplify a product from strawberry, raspberry, or blackberry, and these primers eventually will be very valuable for genome comparison. However, the percentage appears to be too small to be practical in quickly developing SSRs and maps for marker-assisted selection.
Therefore, genome comparisons among species from different Rosaceae subfamilies might be more efficient with hybridization techniques than with PCR-based techniques.

Sequences from any *Fragaria* species appear to be equally useful for designing SSRs for any other *Fragaria* species. Current research projects developing EST sequences from *F. vesca* as a model for studying gene expression in strawberry should yield many repeat-containing sequences from which SSRs can be designed and used with any of the *Fragaria* species. Other research projects developing ESTs from *F. × ananassa*, likewise, should yield widely useful SSRs. The SSRs we tested have limited transference between *Fragaria* and *Rubus*, and these SSRs are particularly interesting for comparative mapping. For example, SSRs associated with resistance to a pathogen in strawberry may be associated with resistance to a similar pathogen in raspberry and blackberry.

Although 30% to 50% of strawberry-derived SSRs amplified a product in raspberry and blackberry, only about 30% to 50% of these detected a polymorphism between parents of the raspberry (‘Jewel’ × NY322) or blackberry (APF-12 × ‘Arapaho’) mapping populations we tested. Therefore, only about 10% of the strawberry-derived SSRs were useful in mapping in these two populations. It seems clear that dependence on strawberry-derived SSRs is insufficient for genetic mapping in *Rubus*, and *Rubus* sequences are required.

Transference among *Rubus* species was not as successful as among *Fragaria* species. SSRs from the Asian (Indonesia and south east China) native noxious weed *R. alcefolius*, a member of the subgenus *Malachobatus*, exhibited only limited cross-species transference to raspberry (subgenus *Ideoebatus*) or to blackberry (subgenus *Rubus*, formerly *Eubatus*). *Rubus* is one of the most diverse genera in the plant kingdom and considerable differentiation of species exists in the three subgenera used in this study (Jennings, 1988). It may be necessary to develop raspberry SSRs for use with raspberry and blackberry SSRs for use with blackberry.

Both BLAST and SSRIT can be used to screen in-house library sequences for SSRs without first having to deposit sequences in GenBank. Other SSR identification tools include two designed to work with the Linux operating system: TROLL (Castello et al., 2002; Source Forge, 2001); and Sputnik available at (Espresso sourceforge.net: 2001). A fourth tool, MISA, present at [Institute of Plant Genetics and Crop Plant Research (IPK) Plant Genome Resources Center, 2002; Thiel et al., 2003] is designed to interface with the Primer3 primer design software with the help of two PERL scripts available on the web site. We compared BLAST and SSRIT and found SSRIT was superior to BLAST, because it identified more GenBank sequences containing repeats and detected a greater diversity of repeat motifs.

SSR primers developed from the genomic library seemed better able to detect polymorphisms among accessions and between parents of mapping populations (Table 3). It is possible that the SSRs derived from the genomic library were more likely to detect polymorphisms because of the larger repeat regions found in the genomic library sequences (Table 1). Among SSRs derived from GenBank accessions, the ability to detect polymorphisms among accessions increased from 43% to 68% as the number of repeats increased from fewer than six repeats to six or more repeats. This suggests SSRs derived from GenBank sequences with six or more repeats would be as likely to detect polymorphisms as would SSRs derived from a genomic library. If a genomic library is not already available, development of SSRs from GenBank sequences may be more efficient than from a genomic or cDNA library.

On the other hand, if the number of sequences deposited in GenBank is insufficient for genetic mapping, development and sequencing of a library is warranted. Since Morgante et al. (2002) showed that SSRs are associated with gene clusters in plants, SSRs developed from cDNA sequences or genomic sequences may be equally likely to be associated with traits. The choice of whether to make a cDNA library or genomic library for SSR development could depend on several factors, including: additional research interests that could be better addressed with one library compared to the other; relative cost of making the two different library types; and technical skills and services available to the researchers involved.

**Literature Cited**


