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An enhanced microsatellite map of diploid *Fragaria*

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Abstract A total of 45 microsatellites (SSRs) were developed for mapping in *Fragaria*. They included 31 newly isolated codominant genomic SSRs from *F. nubicola* and a further 14 SSRs, derived from an expressed sequence tagged library (EST-SSRs) of the cultivated strawberry, *F.* × *ananassa*. These, and an additional 64 previously characterised but unmapped SSRs and EST-SSRs, were scored in the diploid *Fragaria* interspecific F_2 mapping population (FV×FN) derived from a cross between *F. vesca* 815 and *F. nubicola* 601. The cosegregation data of these 109 SSRs, and of 73 previously mapped molecular markers, were used to elaborate an enhanced linkage map. The map is composed of 182 molecular markers (175 microsatellites, six gene specific markers and one sequence-characterised amplified region) and spans 424 cM over seven linkage groups. The average marker spacing is 2.3 cM/marker and the map now contains just eight gaps longer than 10 cM. The transferability of the new SSR markers to the cultivated strawberry was demonstrated using eight cultivars. Because of the transferable nature of these markers, the map produced will provide a useful reference framework for the development of linkage maps of the cultivated strawberry and for the development of other key resources for *Fragaria* such as a physical map. In addition, the map now provides a framework upon which to place transferable markers, such as genes of known function, for comparative mapping purposes within Rosaceae.

Keywords *Fragaria* · Genetic mapping · Microsatellites · EST · Functional genomics

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Introduction

The genus *Fragaria* belongs to the Rosaceae, an economically important family containing many fruit crops (e.g. *Malus*, *Pyrus*, *Prunus* and *Rubus*) and ornamentals (e.g. *Rosa* and *Sorbus*). *Fragaria* is a member of the subfamily Rosoideae and consists of approximately 20 species that are variously diploid, tetraploid, hexaploid or octoploid. These include many wild diploid species such as *F. nubicola* Lindl. and *F. vesca* L., and the cultivated strawberry, *F.* × *ananassa* Duch. ($2n = 8x = 56$).

The cultivated strawberry is an economically important crop plant because of its production of large berries that are grown primarily for the dessert-fruit market. Today growers of *F.* × *ananassa* face increased production challenges which potentially limit profits and sustainable cultivation has traditionally relied on breeding superior cultivars to meet these challenges. Recently there has been a growth of interest in the development of molecular markers such as microsatellites, also known as simple sequence repeats (SSRs), for *Fragaria* that can assist with fingerprinting or the study of the genetic diversity within *Fragaria* germplasm and the breeding

and genetic improvement of the cultivated strawberry (Ashley et al. 2003; Sargent et al. 2003; Cipriani and Testolin 2004; Hadonou et al. 2004; Lewers et al. 2005; Monfort et al. 2005). The majority of these SSR markers have been developed from genomic DNA libraries.

In contrast to SSRs isolated from genomic DNA libraries, those derived from expressed sequence tag (EST) libraries have the advantage of being intrinsically associated with coding sequences within the genome (Eujayl et al. 2002) and thus provide functional information to linkage mapping investigations. The use of EST libraries for the development of polymorphic SSR markers from rosaceous crops has been investigated in apricot (*Prunus armeniaca* L.) (Decroocq et al. 2003) and more recently in peach (*P. persica*), almond (*P. armeniaca*) and rose (*Rosa* spp.) (Jung et al. 2005). However, the utility of these markers to mapping has yet to be demonstrated. Folta et al. (2005) described an EST library constructed from *F.* × *ananassa* and reported that a high proportion of the ESTs sequenced contained SSRs in both the coding and the 3′/5′ untranslated regions.

The first transferable linkage map for *Fragaria* was constructed primarily of genomic SSR markers from an interspecific cross between two closely related and highly interfertile diploid *Fragaria* species, *F. nubicola* and *F. vesca* (Sargent et al. 2004a). Since the diploid and octoploid species share a common genomic base (Senanayake and Bringhurst 1967), the diploid map is a model for the economically important cultivated strawberry. The development of this reference map for the genus will provide a robust framework for a range of map-based investigations, not only in the cultivated strawberry, but also for rosaceous synteny studies. However only 68 SSRs and six gene-specific STS markers have currently been assigned a map position, and the recent markers developed by Lewers et al. (2005), Monfort et al. (2005) and Denoyes-Rothan (unpublished data) from both coding and non-coding DNA sources have yet to be mapped.

In this investigation, we report the development of 45 SSR markers derived from an enriched *F. nubicola* genomic DNA library and from the *Fragaria* EST sequences deposited in GenBank by Folta et al. (2005). In addition to the development of these markers, we report the mapping of these and an additional 64 SSRs developed by Lewers et al. (2005), Monfort et al. (2005) and Denoyes-Rothan (unpublished data) in the *Fragaria* mapping population of Sargent et al. (2004b) and thus, the significant improvement of the diploid *Fragaria* reference map.

Materials and methods

Plant material and DNA extraction

The F_2 mapping population was composed of 77 seedlings from the selfing of EMFX02/03 and 17 seedlings from the selfing of EMFX02/02, two seedlings of the F_1 progeny of a cross of *F. vesca* 815× *F. nubicola* 601. The two sub-populations had the same grandparents and for nearly all loci segregated for the same two alleles. For clarity, this *Fragaria* reference population is referred to as FV×FN to denote its interspecific nature and the species used in its production.

DNA was isolated from young leaf tissue of *F. vesca* 815, *F. nubicola* 601 and 94 seedlings of FV×FN using the DNeasy plant miniprep kit (Qiagen) according to the manufacturer's protocol, and diluted to 1–10 ng/μl for use in PCR. DNA was also extracted from a selection of eight *F.* × *ananassa* cultivars from the East Malling Research cultivar collection to test the transferability of the SSR primer pairs developed in this investigation to the octoploid species.

Microsatellite marker development

Primer pairs were developed for genomic SSRs that were isolated, sequenced and characterised from 608 colony PCRs performed on clones from an enriched library of the *F. nubicola* accession 601 (East Malling Research diploid *Fragaria* germplasm collection) that was developed and cloned according to the procedure reported by Sargent et al. (2003). In addition, from the *F.* × *ananassa* 'Festival' library of Folta et al. (2005), 165 unique SSR-containing sequences were identified, all of which contained an SSR motif of at least $(XX)_5$ or $(XXX)_4$. All novel primer pairs presented in this investigation were designed from DNA sequence flanking the SSR repeats using the software PRIMER 3 (Rozen and Skaletsky 1998). The design criteria included a PCR product between 100 and 350 bp in length, a T_m of 55–65°C (optimum 60°C), and a primer length of 18–27 bp (optimum 20 bp). Genomic SSRs were labelled following the nomenclature of Sargent et al. (2003), and were assigned numbers EMFn101–EMFn238. EST-SSR primer pairs were denoted UFFa (University of Florida *F.* × *ananassa*) followed by the five-character alphanumeric EST code of Folta et al. (2005), i.e. UFFxa 00X00. Along with the primer pairs developed in this investigation, the EST and genomic SSR primer pairs developed by Lewers et al. (2005) (ARSFL/FAC) from *F.* × *ananassa* and those developed by Monfort et al. (2005) (CFVCT) from *F. vesca* were screened for amplification, polymorphism and likely segregation in a subset of the diploid mapping population FV×FN, along with a set of primer pairs developed by Denoyes-Rothan (BFACT) from a *F.* × *ananassa* genomic library (unpublished data). The newly characterised EMFn-SSR marker loci segregating in FV×FN were then tested using the same amplification conditions as for the diploid map construction in eight *F.* × *ananassa* cultivars to assess the transferability of the primer pairs developed to the cultivated strawberry.

PCR conditions and product visualization

All PCRs were performed following the touchdown protocol described by Sargent et al. (2003). PCR products that were generated from both the novel SSRs and those previously described (Lewers et al. 2005; Monfort et al. 2005) in *F. vesca* 815 and *F. nubicola* 601 and a subset of seedlings from FV×FN were initially electrophoresed through an EL600 Spreadex gel (Elchrom) at 75 V for 1 h 30 min which was subsequently stained with SYBR gold for 30 min (Invitrogen) to visualise the products. Primer pairs amplifying polymorphic genotypes were scored in the 94 FV×FN seedlings. Primer pairs amplifying complex patterns of bands were excluded from further investigation.

Segregation was visualised and scored by electrophoresis through an EL600 or EL800 Spreadex gel depending upon expected product sizes (75 V for 1 h 30 min) and staining with SYBR gold (Invitrogen) for 30 min. Markers for which polymorphism could not be scored by Speadex gel electrophoresis were either labelled on the forward primer with one of three fluorescent dyes, 6-FAM, NED or VIC (Applied Biosystems), and the products fractionated by capillary electrophoresis through a 3100 genetic analyser (Applied Biosystems) or the PCR products were separated by electrophoresis on a 6% w/v denaturing acrylamide gel at a constant 65 W for 2–4 h in 1× TBE (Tris–borate, EDTA) buffer and visualized by silver staining (Sambrook et al. 1989). Data generated by capillary electrophoresis were collected and analysed using the GENESCAN and GENOTYPER (Applied Biosystems) software. The polyacrylamide gels were scored visually on two separate occasions by independent investigators.

Data analysis and map construction

Chi-square tests of goodness-of-fit to an expected segregation ratio of 1:2:1 or 3:1 were carried out for all markers segregating in the F_2 seedlings of FV×FN using JOINMAP 3.0 (Van Ooijen and Voorrips 2001). Map positions for the previously unmapped loci were initially assigned using the 'order' and 'try' commands of MAPMAKER 3.0 (Lander et al. 1987), which were verified using the 'ripple' command. Once linkage groups had been assigned, the 'error detect' function was used to detect improbable seedling genotypes for all loci. A final linkage analysis was then performed and the markers were assimilated into the map of Sargent et al. (2004a) using JOINMAP 3.0 applying the Kosambi mapping function. Marker placement was determined using a minimum LOD score threshold of 3.0, a recombination fraction threshold of 0.35, ripple value of 1.0, jump threshold of 3.0 and a triplet threshold of 5.0. The map presented was constructed using MAPCHART for Windows (Voorrips 2002).

Functional annotation of EST sequences

Putative function was assigned to the polymorphic EST-SSRs that were mapped in this investigation through comparison to sequences in known databases using the WU-BLAST2 or FASTA search algorithms and the BLASTX algorithm (Altschul et al. 1990; Gish and States 1993). EST sequences were compared against the AGI, Genbank/SwissPROT and PIR databases, and likely function was inferred from sequences of strongest homology (lowest *E*-value).

Results

Primer design and microsatellite polymorphism

From the enriched *F. nubicola* 601 genomic SSR library, 225 clones were identified from 608 screened that putatively contained an SSR sequence. Sequencing of 217 of these clones revealed 154 unique SSR-containing sequences with a repeat motif of at least $(XX)_5$ or $(XXX)_4$. A total of 137 primer pairs were designed from the 154 genomic DNA SSR sequences obtained from the *F. nubicola* 601 library, of which 31 amplified codominant products that were polymorphic between the parents of FV×FN. A further 46 primer pairs were designed from 165 unique EST-SSR sequences retrieved from the *F.* × *ananassa* EST library of Folta et al. (2005) that contained sufficient flanking DNA and a repeat motif of an appropriate length. Of these, 14 amplified codominant products that were polymorphic between the parental genotypes of FV×FN. The locus names, primer sequences, repeat motifs and expected product sizes of these 45 polymorphic SSR markers are presented in Table 1.

All 45 SSR primer pairs developed in this investigation that segregated in FV×FN amplified PCR products in the eight *F.* × *ananassa* cultivars screened. When separated on an EL800 Spreadex gel, distinct banding patterns were observed amongst cultivars, indicating putative polymorphism and thus the potential of these markers for mapping in the cultivated strawberry.

A total of 32 primer pairs described by Monfort et al. (2005) amplified products of the expected size in the parents of FV×FN. Seventeen amplified a codominant product, 14 amplified a dominant product only in the *F. vesca* 815 parent and a single primer pair amplified a dominant product only in the *F. nubicola* 601 parent of FV×FN. From the primer pairs of Lewers et al. (2005), 18 amplified a codominant, polymorphic product segregating in FV×FN and a further 14 primer pairs, 13 codominant and one dominant (Denoyes-Rothan unpublished data), amplified a product polymorphic between *F. vesca* 815 and *F. nubicola* 601.

Table 1 The locus names, primer sequences (F = forward, R = reverse), repeat motifs and cloned fragment sizes for 45 codominant, polymorphic SSR markers developed from an SSR-enriched genomic DNA library of *F. nubicola* together with EMBL accession numbers

Marker name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Expected size	Motif	EMBL accession number
Genomic SSRs					
EMFn 110	GACGCTTCGGAGACTGAGG	CCCCCTTAAAAATAATTAAATCTCC	231	$(CT)_9$	AM051323
EMFn 115	TGGAGATGATGGTCAAGACG	GACAAGACCACGAAAACACG	221	$(AG)_9...(AG)_9$	AM051324
EMFn 117	ATCGGATCAACAAGCAAAGC	ATGGATGAGGGGAGAAGAGG	175	$(CT)_{15}$	AM051325
EMFn 119	ATGGCGGAGAGGAGTTGG	CATCGAACTTATGGGGTTGC	138	$(GA)_{28}$	AM051326
EMFn 121	GGTCCCTAAGTCCATCATGC	GAGTGGATGCAAACATGAGC	244	$(GT)_{12}...(GA)_9$	AM051327
EMFn 123	CATTTCGGGCACACTTCC	AGACGGCAAAGAGACTCACC	219	$(CT)_{24}$	AM051328
EMFn 125	CCCAACCCTAAACCATACCC	ATGGTTGCCTTTGATTCACG	222	$(CT)_8$	AM051329
EMFn 128	CATCAACATTCACATGAATTTACC	CGGCGGATCTAGTTTTGAGG	191	$(CT)_{25}$	AM051330
EMFn 134	TGATTCTTTGAAAGGCTTTGG	AAAACAACCCCCTCTCATCC	213	$(AG)_{11}$	AM051331
EMFn 136	TTTCTCTTTTGCTCCATAGTTCC	TTCATCAGGATCCAGAAGTCC	242	$(CT)_{14}$	AM051332
EMFn 148	TTACCTGCACAGAAACAACG	CAACTTCCTCCTCACTCACC	239	$(AG)_6...(AG)_{11}$	AM051333
EMFn 150	CAAGTCTCTCTCGGTGTTTCG	AAACCTGTGTGGACAAGATCC	137	$(AG)_{14}$	AM051334
EMFn 152	GGGCCAAAATGAGTATCTTGC	TTAGAGCGAGGTGGTAATGC	155	$(GA)_{13}$	AM051335
EMFn 153	CTCGAGCTCCCTTTCTATCG	TGGCCAAATGTTCTCACTAGC	246	$(CT)_{13}$	AM051336
EMFn 160	GCATCCTTGGGAAATTAATGC	TTGGGAAGGATCATAAAAACC	219	$(CT)_{24}$	AM051337
EMFn 162	AACTGTGTGGTATGCATGTAGC	ATGCACAAAGCAATGCAAGC	231	$(CT)_{10}$	AM051338
EMFn 170	CAGTTTGCCCAACAACAAGG	TTGATGGCAACAAATCACG	192	$(CT)_9$	AM051339
EMFn 181	CCAAATTCAAATTCCTCTTTCC	GCCGAAAAACTCAAACTACCC	212	$(AG)_{37}$	AM051340
EMFn 182	GCAACAAAGGAGGTTAGAGTCG	TGGTGAGTGCTCATTGTTCC	179	$(GT)_8$	AM051341
EMFn 184	GATGAGAATTGTTTGAGTGAAGG	TGACCAGCGGATTCATAAGG	238	$(CT)_{10}$	AM051342
EMFn 185	GTAACGACGGCTGCTTCTCC	CGCTCGCTCTTATAAACTTCC	217	$(GA)_{11}$	AM051343
EMFn 198	CCAAATTGTCCTTGATGTCG	CACCTGCTTCAAAGCAAACC	181	$(CT)_{24}$	AM051344
EMFn 201	CAGCTCAGAAAAGCTCACAGC	TAGAACGCCAATCACAAACC	211	$(AG)_7$	AM051345
EMFn 202	CTCTCTCCCTCAACCTCTCG	TGGACCAATATCTCCCTTGC	229	$(CT)_{13}$	AM051346
EMFn 207	TTGGCAAGAATTTATAGCATCG	TCAGGATGTCTTCAGCAAGG	239	$(AG)_{12}$	AM051347
EMFn 213	AGCGTGATTTTGCCTTTGTT	CACAGTAAAGAACAGGAGGGAGAT	350	$(CT)_{24}$	AM051348
EMFn 214	CTAATTCAGCCGCCAGGTC	CTGCAGCTCGTAACGACAAG	293	$(CT)_9$	AM051349
EMFn 225	AAGGAAAAATGCTCAAATACCC	TACGTGCGACGTTAGAGTCC	274	$(CT)_{21}$	AM051350
EMFn 226	CGTCAAAGGAACCCTATTTCG	GTGACGGAGGCATCTTGG	240	$(CT)_{11}$	AM051351
EMFn 228	TTGCTGAGGATTTGAAAATGG	TGAAGTTTACTGGCGTTTGC	254	$(CT)_9$	AM051352
EMFn 230	AATGACTACGACAACGACAGTCT	AGGGAAAATGCCCAAATACC	273	$(GA)_{15}...(GA)_{13}$	AM051353
EMFn 235	AGGAACAAGAGCTGGCAATG	CTCAAGTATCAGGCCTCCAAG	207	$(GT)_{10}...(GA)_{12}$	AM051323
EMFn 238	TTTACTACAGAGCTGAAGCTACCC	GAAGAAGCCCATTATCAGAAGC	296	Complex	AM051324
EST-SSRs					
UFFa 01E03	ACCCCATCTTCTTCAAATCTCA	GACAAGGCCAGAGCTAGAGAAG	185	$(CAC)_{10}$	AJ870458
UFFa 01H05	GGGAGCTTGCTAGCTAGATTTG	AGATCCAAGTGTGGAAGATGCT	246	$(CT)_8$	AJ870459
UFFa 02F02	CTTTGCAGCTGAAGAACTCTGA	CAGCAGCTGCCTTAGTCTTAGT	199	$(AGG)_3...(AGA)_5$	AJ870441
UFFa 02H04	ATCAGTCATCCTGCTAGGCACT	TACTCTGGAACACGCAAGAGAA	202	$(TCG)_6$	AJ870442
UFFa 03B05	GGAATCCAAGTTACAGGCTTCA	AAGGAGCCTCTCCAATAGCTTC	231	Complex	AJ870443
UFFa 04G04	ACGAGGCCTTGTCTTCTTTGTA	GCTCCAGCTTTATTGTCTTGCT	187	$(TTC)_7$	AJ870445
UFFa 08C11	GGACGTCCCCTTCTTTATTTCT	ACCCCACATTCCATACCACTAC	203	$(TGG)_6$	AJ870446
UFFa 09B11	CTTGGGAGAGAACCAGAAAAAC	TCAGAACCAACTCCAGAGAAGC	197	$(AG)_6$	AJ870448
UFFa 09E12	CGAGGAAGTAACCTCACAGAAA	GGTGATGGAGAGTGCTGTTAGA	193	$(AC)_6$	AJ870449
UFFa 09F09	AGAACCATCATCGTCTCTCGTT	GCAATCTCTTCCGGCTTAAACT	218	$(AAG)_3...(AAG)_6$	AJ870450
UFFa 15H09	TTAGTAGTAGACCTGCCACAAGG	CGGCTTATCTGTAGAGCTTCAA	228	$(CAGAG)_6$	AJ870452
UFFa 16H07	CTCTACCACCATTCAAAACCTC	CACTGGAGACATCTAGCTCAAAC	248	$(CT)_{11}$	AJ870453
UFFa 19B05	GACGAGTTAACATCAACGACAC	TACTTAGGCTGCTGCTCTATCTG	232	$(CAT)_9$	AJ870454
UFFa 20G06	ACTCAACCACCACATTTCACAC	GAGAAGTTGTCAATAGTCCAGGTG	154	$(CT)_{11}$	AJ870457

Microsatellite mapping in FV×FN

Significant deviation from the expected 1:2:1 and 3:1 ratios was detected at 64 of the 109 (59%) novel loci scored in FV×FN. The segregation data and chi-square values for goodness-of-fit to the expected Mendelian segregation ratios for the 109 newly scored polymorphic markers, along with the linkage group to which they located, are given in Table 2. All 109 marker loci that segregated were mapped in FV×FN and these markers located to the seven diploid linkage groups (LG) (Fig. 1). These segregation data have been placed in the Genome Database for Rosaceae (Jung et al. 2004) and the interactive version of the FV×FN reference map (http://www.genome.clemson.edu/gdr/cmap/) has been updated to incorporate these new loci. The error detection function of MAPMAKER 3.0 (Lander et al. 1987) highlighted a number of scoring errors in the previous data of Sargent et al. (2004a) which were corrected from the original segregation data before the final linkage analysis was performed.

Overall, the placement of 109 previously unmapped SSR loci onto the diploid *Fragaria* reference map increased the total number of markers mapped to 182. The linkage map now comprises some 175 microsatellites, one sequence-characterised amplified region and six gene specific markers. Some of the loci placed on the map located to, and extended the ends of, the original FV×FN linkage groups, e.g. UFFxa03B05 (LGII), CFVCT012 (LGIII), CFVCT005a (LGIV) and

Table 2 Monogenic segregation data and chi-square values for goodness-of-fit to expected Mendelian segregation ratios 1:2:1 (aa:ab:bb), 3:1 (a_:bb) or 1:3 (aa:b_), where the a-allele is that from *F. vesca* 815 and the b-allele is that from *F. nubicola* 601, for the 109 markers scored in FV×FN detailing linkage group (LG) to which the markers located. Segregation ratios deviating significantly from the expected ratios ($P \leq 0.05$, 0.01, 0.001) are indicated with one, two and three asterisks, respectively

Locus	Expected	Observed	χ^2	Df	Significance	Linkage group
ARSFL-009	1:2:1	15:52:27	4.1	2	–	III
ARSFL-010	1:2:1	23:51:20	0.9	2	–	I
ARSFL-011	1:2:1	19:35:40	15.5	2	***	VII
ARSFL-012	1:2:1	21:33:39	14.8	2	***	II
ARSFL-013	1:2:1	29:41:24	2.1	2	–	I
ARSFL-014	1:2:1	15:51:27	4	2	–	III
ARSFL-015	1:2:1	10:46:38	16.7	2	***	II
ARSFL-017	1:2:1	21:52:21	1.1	2	–	II
ARSFL-022	1:2:1	24:55:15	4.5	2	–	VI
ARSFL-024	1:2:1	19:32:41	19	2	***	VII
ARSFL-027	1:2:1	16:52:26	3.2	2	–	III
ARSFL-031	1:2:1	6:39:45	35.4	2	***	II
ARSFL-092	1:2:1	23:42:22	0.1	2	–	I
ARSFL-099	1:2:1	22:43:28	1.3	2	–	VII
BFACT-002	1:2:1	7:41:40	25.2	2	***	II
BFACT-004	1:2:1	18:34:42	19.4	2	***	VII
BFACT-008	1:2:1	13:44:36	11.7	2	***	IV
BFACT-010	3:1	79:5	16.3	1	***	VI
BFACT-018	1:2:1	10:37:27	7.8	2	**	VII
BFACT-029	1:2:1	18:31:42	21.9	2	***	VII
BFACT-031	1:2:1	22:45:27	0.7	2	–	VII
BFACT-036	1:2:1	16:49:26	2.7	2	–	III
BFACT-039	1:2:1	23:50:21	0.5	2	–	II
BFACT-042	1:2:1	18:38:29	3.8	2	–	I
BFACT-043	1:2:1	19:40:23	0.4	2	–	III
BFACT-044	1:2:1	20:43:25	0.6	2	–	VII
BFACT-045	1:2:1	17:53:24	2.6	2	–	III
BFACT-047	1:2:1	23:56:12	7.5	2	**	VI
CFVCT002	3:1	79:13	5.8	1	**	VI
CFVCT003	1:2:1	14:42:34	9.3	2	***	V
CFVCT004	1:2:1	21:50:18	1.6	2	–	I
CFVCT005A	3:1	74:15	3.1	1	*	IV
CFVCT005B	3:1	71:20	0.4	1	–	I
CFVCT006	3:1	54:35	9.7	1	***	IV
CFVCT007	1:2:1	18:43:25	1.1	2	–	III
CFVCT009	3:1	53:34	9.2	1	***	VII
CFVCT010	3:1	79:14	4.9	1	**	VI
CFVCT011	3:1	68:24	0.1	1	–	III
CFVCT012	3:1	55:37	11.4	1	***	III
CFVCT014	1:2:1	12:45:36	12.5	2	***	IV
CFVCT015	1:2:1	5:40:45	36.7	2	***	II
CFVCT016	1:2:1	15:41:33	7.8	2	**	V
CFVCT017	1:2:1	21:52:17	2.5	2	–	VI
CFVCT019	3:1	61:30	3.1	1	*	VII
CFVCT020	3:1	44:47	34.5	1	***	II
CFVCT021	1:2:1	28:43:18	2.4	2	–	II
CFVCT022	1:2:1	16:47:28	3.3	2	–	III
CFVCT023	1:2:1	21:42:25	0.6	2	–	VII
CFVCT024	1:2:1	16:41:35	8.9	2	**	V
CFVCT025	3:1	45:36	16.3	1	***	II
CFVCT026	1:2:1	19:32:42	20.4	2	***	VII
CFVCT027	1:2:1	2:44:45	40.7	2	***	II
CFVCT028	1:2:1	9:65:12	22.7	2	***	VI
CFVCT030	1:2:1	23:54:15	4.2	2	–	VI
CFVCT031A	3:1	51:40	17.4	1	***	VII
CFVCT031B	3:1	44:47	34.5	1	***	II
CFVCT031C	1:3	18:73	1.3	1	–	VII
CFVCT032	1:2:1	9:58:25	11.8	2	***	III
CFVCT035	3:1	67:26	0.4	1	–	III
CFVCT036	1:2:1	22:55:15	4.6	2	–	VI
EMFn110	1:2:1	14:46:33	7.8	2	**	V
EMFn115	1:2:1	22:51:20	1	2	–	I
EMFn117	1:2:1	25:58:9	11.8	2	***	VI
EMFn119	1:2:1	25:54:15	4.2	2	–	VI

Table 2 (Contd.)

Locus	Expected	Observed	χ^2	Df	Significance	Linkage group
EMFn121	1:2:1	2:44:47	43.8	2	***	II
EMFn123	1:2:1	25:59:9	12.2	2	***	VI
EMFn125	1:2:1	17:51:26	2.4	2	–	III
EMFn128	1:2:1	25:49:20	0.7	2	–	I
EMFn134	1:2:1	11:48:35	12.3	2	***	II
EMFn136	1:2:1	18:43:33	5.5	2	*	I
EMFn148	1:2:1	3:42:49	46.1	2	***	II
EMFn150	1:2:1	3:43:48	43.8	2	***	II
EMFn152	1:2:1	23:49:20	0.6	2	–	I
EMFn153	1:2:1	22:49:23	0.2	2	–	VI
EMFn160	1:2:1	28:45:21	1.2	2	–	II
EMFn162	1:2:1	12:43:38	15.1	2	***	V
EMFn170	1:2:1	16:50:27	3.1	2	–	III
EMFn181	1:2:1	13:47:34	9.4	2	***	V
EMFn182	1:2:1	26:42:26	1.1	2	–	I
EMFn184	1:2:1	13:46:35	10.3	2	***	V
EMFn185	1:2:1	28:58:8	13.7	2	***	VI
EMFn201	1:2:1	19:35:39	14.3	2	***	VII
EMFn202	1:2:1	16:52:26	3.2	2	–	III
EMFn207	1:2:1	16:58:20	5.5	2	*	III
EMFn213	1:2:1	18:42:34	6.5	2	**	VII
EMFn214	1:2:1	9:46:37	17	2	***	II
EMFn225	1:2:1	23:61:8	14.7	2	***	VI
EMFn226	1:2:1	18:51:25	1.7	2	–	III
EMFn228	1:2:1	39:46:4	27.6	2	***	VI
EMFn235	1:2:1	2:45:44	38.8	2	***	II
EMFn238	1:2:1	13:47:34	9.4	2	***	V
FAC-001	1:2:1	18:33:42	20.2	2	***	VII
FAC-004d	1:2:1	31:56:7	15.7	2	***	VI
FAC-005	1:2:1	20:50:22	0.8	2	–	VI
FAC-012a	1:2:1	23:39:19	0.5	2	–	VI
UFFxa01E03	1:2:1	15:54:25	4.2	2	–	VI
UFFxa01H05	1:2:1	12:44:37	13.7	2	***	IV
UFFxa02F02	1:2:1	25:46:23	0.1	2	–	I
UFFxa02H04	1:2:1	19:45:30	2.7	2	–	III
UFFxa03B05	1:2:1	45:9:34	58.4	2	***	II
UFFxa04G04	1:2:1	12:45:37	13.5	2	***	IV
UFFxa08C11	1:2:1	3:41:48	45.1	2	***	II
UFFxa09B11	1:2:1	6:41:47	37.3	2	***	II
UFFxa09E12	1:2:1	2:42:48	46.7	2	***	II
UFFxa09F09	1:2:1	12:47:35	11.3	2	***	II
UFFxa15H09	1:2:1	13:46:35	10.3	2	***	II
UFFxa16H07	1:2:1	25:44:24	0.3	2	–	I
UFFxa19B10	1:2:1	18:33:42	20.2	2	***	VII
UFFxa20G06	1:2:1	17:32:42	21.8	2	***	VII

CVFCT009/BFACT044 (LGVII). However, because of the removal of a number of errors in the original mapping data, the total length of the map presented herein was reduced by 24 cM (5%) to 424.3 cM. In addition, there were minor rearrangements in the placement of some of the dominant or highly skewed marker loci presented by Sargent et al. (2004a) due to a better estimation of mapping distances with the addition of the novel SSR loci. That none of the loci scored remained unlinked indicates that the FV×FN reference map provides comprehensive coverage of the diploid *Fragaria* genome with an average density of one marker every 2.3 cM.

The placement of the 109 SSR markers closed up many of the large gaps over 10 cM in length on the previous map, reducing their number from 15 to 8. However, a region of marker clustering was still apparent on each of the seven linkage groups. Marker EMFn136 located between F3H and EMFvi072 at the top of LGI, increasing the support for the linkage in this area from a LOD of 2.47 to a LOD in excess of 8.0. The markers mapped in this investigation were distributed across all seven linkage groups; however, marker coverage of LGII increased the most, with the addition of 25 markers, whilst just six new markers were placed on LGIV, one of which, CFVCT005a, extended the end of the linkage group by just over 25 cM. As in the previous mapping investigation of Davis and Yu (1997), LGII was the longest linkage group, covering a distance of 79.3 cM, whilst LGVII was the shortest, covering a distance of 43.1 cM. The average length of the seven linkage groups on the FV×FN map was 60.6 cM, with an average of 26 markers per linkage group.

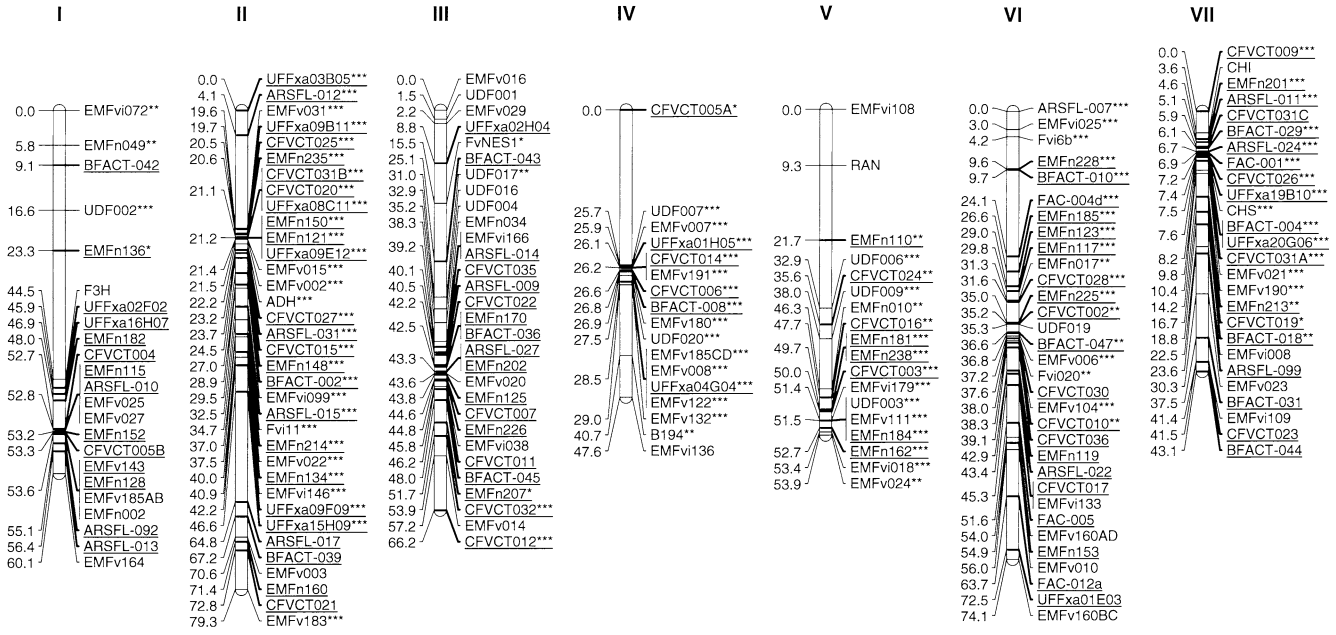

Fig. 1 A genetic linkage map of 182 markers in seven linkage groups constructed from an F_2 progeny from the cross of *F. vesca* 815× *F. nubicola* 601 (FV×FN) showing the map positions of 31 newly characterised genomic DNA-derived SSRs, 14 EST-SSR loci and 64 previously published but unmapped SSRs, along with the 73 molecular markers previously reported by Sargent et al. (2004a). Loci mapped in this investigation are given *underlined*. Mapping distances are given in centiMorgans (cM). Markers with segregation ratios deviating significantly from the expected ratios ($P \leq 0.05$, 0.01, 0.001) are indicated with one, two or three asterisks, respectively

Putative EST function

Of the 14 EST-SSRs located on the *Fragaria* reference map, ten showed homology to gene sequences deposited in EMBL (Table 3). Of the remaining four, three (UFFxa 08H09, UFFxa 09B11 and UFFxa 09E12) displayed partial homology to genes or proteins in the databases. Only one, UFFxa 15H09, showed no homology to any previously characterised genes or DNA sequences.

Discussion

Microsatellite mapping in FV×FN

We have reported the isolation and characterisation of 31 co-dominant, polymorphic SSR markers derived from a *F. nubicola* genomic DNA library along with an additional 14 co-dominant EST-SSRs. These markers, and a further 64, previously described but unmapped, *Fragaria* SSRs (Lewers et al. 2005; Monfort et al. 2005; Denoyes-Rothan unpublished data) have been assimilated onto the diploid *Fragaria* reference map of Sargent et al. (2004a), greatly increasing its marker density from one marker every 5.9 cM to one marker every 2.3 cM. The mapping of these 109 SSR markers has significantly increased the overall number of markers on the diploid *Fragaria* reference map to 182 (149%) and has more than doubled the number of SSR loci of the previous map of diploid *Fragaria* (Sargent et al. 2004a).

On all seven linkage groups, a distinct region of marker clustering was observed. Arenshchenkova and Ganal (1999) showed that clustering of microsatellites occurred in tomato, where they were most predominantly associated with the centromeres. Centromeric microsatellite clustering has also been reported in such diverse species as *Phaseolus* (Blair et al. 2003), *Helianthus* (Tang et al. 2002) and *Pinus* (Elsik and Williams 2001). In barley (*Hordeum vulgare*), where distinct centromeric clustering has also been observed (Li et al. 2003; Künzel et al. 2000; Ramsay et al. 2000), it was suggested that it was due to a non-random physical distribution of SSR repeats throughout the genome. Ramsay et al. (2000) suggested that suppressed recombination around the centromeres in barley can be accentuated in wide crosses. Such a phenomenon may also affect interspecific crosses in *Fragaria* and may contribute to the clustering of markers observed here on the *Fragaria* reference map.

PCR-based systems such as AFLPs and RAPDs (Vos et al. 1995; Welsh and McClelland 1990; Williams et al. 1990) coupled with targeted approaches to SSR development (Cregan et al. 1999; Wang et al. 2002) or the development of SCARs (Paran and Michelmore 1993) could be used for the development of markers to populate the areas of the diploid *Fragaria* reference map where there is currently a low density of molecular loci, such as on LGIV.

Table 3 Genes to which the 14 mapped *Fragaria* × *ananassa* ESTs showed greatest homology and their putative function deduced from BLAST/FASTA homology for the 14 loci mapped in FV×FN, along with the gene reference numbers for the sequences with which they showed the greatest homology and the species from which these sequences were derived where they were not *Arabidopsis* homologues

Marker name	Nearest *Arabidopsis* homolog	Gene function in species from which it was characterized	Gene reference	*E*-value
UFFxa 01E03	DegP protease	Belongs to a closely-related family of chloroplast proteins with processing functions in the lumen (Chassin et al. 2002)	At3g27925	1.0e-37
UFFxa 01H05	Similarity to pollen major allergen 2 protein (*Juniperus ashei*)	Possible role in food allergies.	At3g22820	1.2e-27
UFFxa 02F02	Similar to histone H1-3, (*Lycopersicon pennellii*)	Specialized linker histone that appears to alter chromatin in response to stress (Ascenzi and Gantt 1997).	At2g18050	3.8e-1
UFFxa 02H04	ERD4 protein	Corresponds to a gene of unknown function that is inducible by drought stress (Taji et al. 1999).	At4g04340	4.7e-27
UFFxa 03B05	Response regulator 7 (ARR7)	Protein similar to prokaryotic response regulators; function in regulating the progression of the circadian oscillator (Yamamoto et al. 2003).	At1g19050	5.2e-20
UFFxa 04G04	Nucleotide sugar epimerase	Enzyme involved in nucleotide sugar interconversion pathways; has important roles in activating monosaccharides for alterations of polysaccharides in cell wall, glycolipids and glycoproteins (Reiter and Vanzin 2001).	At3g23820	1.6e-53
UFFxa 08C11	Proline-rich extensin-like protein	Hydroxyproline-rich glycoproteins that increase the strength of cell walls (Kieliszewski and Lamport 1994). Expression linked to wounding and plant defence responses (Zhou et al. 1992).	At1g76930	1.8e-19
UFFxa 09B11	Putative glycine-rich protein	Uncharacterised protein with only partial homology detected in other plant species	At4g29020	3.8e-12
UFFxa 09E12	Expressed protein of unknown function	Uncharacterised protein with only partial homology detected in other plant species	At2g15960	0.0082
UFFxa 09F09	Profilin 5, PRO5 (PRF3)	Actin binding proteins central to polymerisation of actin filaments, involved in cytoplasmic streaming, cytokinesis and elongation in pollen tubes and root hairs (Deeks et al. 2002).	At5g56600	1.3e-47
UFFxa 15H09	No significant homology		N/A	N/A
UFFxa 16H07	bZIP transcription factor	bZIP family of transcriptional regulators have diverse roles in physiology, regulating responses to light, drought, hormones, stress and pathogens (Jakoby et al. 2002).	At4g34590	3.7e-16
UFFxa 19B10	bZIP transcription factor	bZIP family of transcriptional regulators have diverse roles in physiology, regulating responses to light, drought, hormones, stress and pathogens (Jakoby et al. 2002).	At4g34590	2.8e-10
UFFxa 20G06	Ubiquitin-specific protease 12	Class of enzyme with role in function of the 26S proteosome pathway, cleaving the bond between the C-terminal glycine of ubiquitin and its conjugated substrate (Yan et al. 2000)	At5g06600	3.7e-42

ESTs as a source of microsatellites

Expressed sequence tag libraries have been shown to be a good source of SSRs in many species and the 8.9% of SSR-containing EST sequences revealed from the library of Folta et al. (2005) compares favourably with similar studies in other species e.g. kiwi (*Actinidia* spp.) (3.1%) (Fraser et al. 2004), white clover (*Trifolium repens* L.) (7%) (Barrett et al. 2004) and pepper (*Capsicum* spp.) (10.5%) (Sanwen et al. 2000). EST-SSRs have been reported to be less polymorphic than those developed from genomic DNA in clover (Barrett et al. 2004). However, in this investigation, they proved to be highly polymorphic (14/46 markers). Markers derived from EST libraries have the advantage of greater biological significance over SSRs derived from non-coding genomic DNA. EST-SSRs identified in economically important genes are more useful markers for the purposes of

marker-assisted selection, as intragenic recombination occurs only rarely and thus, for the purposes of breeding, SSR alleles can be associated with a high degree of certainty with trait-influencing mutations in the genes concerned. Development of microsatellites from existing ESTs has the additional advantage of large savings in laboratory time and costs over development of genomic DNA-derived SSRs. The SSR-containing EST sequences are freely available from nucleotide sequence databases and thus the development of primer pairs for the 14 EST-SSRs took 2–3 days, compared to up to 8 weeks for the 31 SSRs derived from the genomic DNA library.

Assigning function to the EST-SSRs

Some of the EST-SSRs derived from the EST library of Folta et al. (2005) and mapped in FV×FN are associated with genes of potential commercial importance, including a strawberry profilin (UFFa 09F09) and a protein with homology to major allergen 2 in juniper (*Juniperus* spp.) (UFFa 01H05). Other loci mapped represent genes of diverse biological function in other species, from those with roles in plant defence responses, cytokinesis and ubiquitination to transcription factors. That UFFa 15H09 showed no significant homology with any genes or proteins in the databases is consistent with the finding that some of the library sequences are currently unique to strawberry (Folta et al. 2005). The 71% (10/14) of mapped EST-SSR markers that showed strong homology to genes of known function in this investigation is comparable with the homology to genes displayed by ESTs from almond (71%), peach (79%) and rose (71%) (Jung et al. 2005). With the placement of the EST-SSR markers with assigned putative function on the *Fragaria* reference map along with those of Lewers et al. (2005) and Monfort et al. (2005) and their highly transferable nature, the roles of the genes with which they are physically associated can be studied in both the diploid and octoploid genetic backgrounds. In this way, they will provide valuable tools for a candidate gene approach to elucidate the underlying mechanisms of important morphological and physiological traits within strawberry.

Utility to mapping in other species

The SSR markers developed in this investigation all amplified putatively polymorphic products of the expected size in the *F.* × *ananassa* germplasm screened, consistent with the transferability of other SSR markers developed for *Fragaria* (Hadonou et al. 2004) and indicating the generic utility of these markers to mapping studies and fingerprinting in the genus *Fragaria*. Although approximately half the loci mapped in this investigation displayed distorted segregation ratios, similar levels of distortion have also been observed in the intraspecific diploid *Fragaria* map of Davis and Yu (1997) and have previously been discussed by Sargent et al. (2004a). Despite the distortion observed, preliminary studies in the octoploid *F.* × *ananassa* have identified four homologues of each of the seven linkage groups presented herein and marker order within these groups is largely conserved (Denoyes-Rothan unpublished data). Thus the diploid map is representative of *Fragaria*.

In addition to serving as a reference map for the genus, there is also sufficient saturation of the genome to permit the construction of reduced maps of other diploid *Fragaria* populations for the mapping of specific qualitative and quantitative morphological traits (Aranzana et al. 2003). It will also allow synteny studies of Rosoideae with Prunoideae (*Prunus*) and Maloideae (*Malus*) to be performed.

Concluding remarks

We have described the development of 45 SSRs for *Fragaria* and have reported the map positions of these markers, along with 64 previously published SSRs on the diploid *Fragaria* reference map. In addition, we have assigned putative function to the majority of the 14 EST-SSRs mapped by comparison of the EST sequences from which they were derived to gene and protein sequences of known function from other species. The working map represents significant progress with respect to the previously published linkage map of Sargent et al. (2004a). Ultimately, the transferable nature of the SSR markers employed in the construction of this diploid reference map will facilitate their application to genetic maps of *F.* × *ananassa* and will provide a basis for marker-assisted selection and functional genomics within the genus.

This reference map will provide a platform for the development of genomics resources for *Fragaria*. Because of its interspecific nature, this map will facilitate the mapping of known function genes, which have been shown to be significantly less polymorphic in intraspecific *F. vesca* mapping populations (Deng and Davis 2001). Thus, this map constitutes a framework upon which to place markers for known-function genes for the study of synteny between strawberry and the other rosaceous genera.

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