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ORIGINAL PAPER

A microsatellite linkage map for the cultivated strawberry (*Fragaria* × *ananassa*) suggests extensive regions of homozygosity in the genome that may have resulted from breeding and selection

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Abstract The linkage maps of the cultivated strawberry, *Fragaria* × *ananassa* ($2n = 8x = 56$) that have been reported to date have been developed predominantly from AFLPs, along with supplementation with transferrable microsatellite (SSR) markers. For the investigation of the inheritance of morphological characters in the cultivated strawberry and for the development of tools for marker-assisted breeding and selection, it is desirable to populate maps of the genome with an abundance of transferrable molecular markers such as microsatellites (SSRs) and gene-specific markers. Exploiting the recent release of the genome sequence of the diploid *F. vesca*, and the publication of an extensive number of polymorphic SSR markers for the genus *Fragaria*, we have extended the linkage map of the 'Redgauntlet' × 'Hapil' (RG × H) mapping population to include a further 330 loci, generated from 160 primer pairs, to create a linkage map for *F.* × *ananassa* containing 549 loci, 490 of which are transferrable SSR or gene-specific markers. The map covers 2140.3 cM in the expected 28 linkage groups for an integrated map (where one group is composed of two separate male and female maps), which represents an estimated 91% of the cultivated strawberry genome. Despite the relative saturation of the linkage map on the majority of linkage groups, regions of apparent extensive homozygosity were identified in the genomes of 'Redgauntlet' and 'Hapil' which may be indicative of allele fixation during the breeding and selection of modern *F.* × *ananassa* cultivars. The genomes of the octoploid and diploid *Fragaria* are largely collinear, but through comparison of mapped markers on the RG × H linkage map to their positions on the genome sequence of *F. vesca*, a number of inversions were identified that may have occurred before the polyploidisation event that led to the evolution of the modern octoploid strawberry species.

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Introduction

The cultivated strawberry, *Fragaria* × *ananassa* ($2n = 8x = 56$) is a crop of major significance and is the most economically important berry crop worldwide, with global production in 2009 in excess of 4.1 million tonnes, which was valued at over US $4 billion (FAOStat; http://faostat.fao.org/). Improved fruit quality, yield, extended storage capability and disease resistance, amongst other traits, are the key priorities of cultivated strawberry genetic improvement programmes worldwide, the breeding of which could be significantly enhanced using marker-assisted breeding and selection (MABS). However,

F. × *ananassa* has a complex polyploid genome ($2n = 8x = 56$) with probable contributions from up to four diploid ancestors (Rousseau-Gueutin et al. 2009). A number of cytological genome models have been proposed for the octoploid species (Fedorova 1946; Senanayake and Bringhurst 1967), but the most widely accepted to date is that of Bringhurst (1990), who suggested the conformation $AAA'A'BBB'B'$. This reflects the belief that the octoploid *Fragaria* genome has undergone diploidisation and that inheritance proceeds in a predominantly disomic fashion. As with other crop plants of polyploid origin such as cotton, wheat and oilseed rape (Liu et al. 2001; Parkin et al. 2005; Wang et al. 2011), the complex nature of the cultivated strawberry genome has made the genetic analysis of quantitative traits within the species, and thus the development of markers for MABS, a challenging process.

An understanding of the genome structure of the cultivated strawberry at the molecular level is an essential prerequisite to the identification of molecular markers linked to agronomic traits within the species, and the development of well-characterised linkage maps is crucial to this process. Additionally, linkage maps are important tools for several other purposes including the investigation of genome evolution and the map-based cloning of genes. A number of linkage maps have been developed for *F.* × *ananassa*, using various molecular marker systems including amplified fragment length polymorphisms (AFLPs), randomly amplified polymorphic DNAs (RAPDs), microsatellites (SSRs) and gene-specific (STS) markers. These maps have characterised the genome of the cultivated strawberry to varying degrees of completeness (Lerceteau-Kohler et al. 2003; Sugimoto et al. 2005; Rousseau-Gueutin et al. 2008; Weebadde et al. 2008; Sargent et al. 2009; Zorrilla-Fontanesi et al. 2011a). In addition to maps of *F.* × *ananassa*, linkage maps have also been developed for other *Fragaria* species, including *F. virginiana* ($2n = 8x = 56$), a wild progenitor of the cultivated strawberry (Spigler et al. 2008) and a map of an interspecific F_2 cross between two diploid species, *F. vesca* × *F. bucharica* ($2n = 2x = 14$) which has been established as the reference map for the genus (Sargent et al. 2006). The reference map contains a total of 411 sequence characterised (STS) markers mapped in the full mapping progeny of 76 individuals (Sargent et al. 2011), and a further 298 STS markers located to the map using a selective or 'bin' mapping progeny of six individuals (Sargent et al. 2008; Illa et al. 2011; Zorrilla-Fontanesi et al. 2011b). The map spans a total of seven linkage groups and covers a genetic distance of 442.8 cM (Sargent et al. 2011).

Recently, the genome sequence of a wild diploid relative of the cultivated strawberry, *F. vesca* 'Hawaii 4' (FvH4) was sequenced in its entirety, and the genome sequence was anchored to the diploid *Fragaria* reference map (Shulaev et al. 2011; Sargent et al. 2011). A total of 219 Mb of sequence data were sequenced to an average depth of coverage of 39× and assembled into approximately 3,200 sequence scaffolds. To these scaffolds, 34,809 predicted genes were located, identified through deep transcriptome sequencing, and 25,050 of them were assigned preliminary annotation. Of the 272 sequencing scaffolds over 50 kbp in length that contained the majority of the sequence data (209.3 Mb; 96% total sequence data), 222, containing a total of 197.7 Mb of nucleotides have been anchored to precise positions on the diploid *Fragaria* genetic map using the 411 STS markers positioned on that map (Sargent et al. 2011). Thus, these 222 sequencing scaffolds have been assigned to one of seven pseudomolecules representing the haploid chromosomes of the *F. vesca* genome.

Linkage mapping investigations in the cultivated strawberry have identified major genes and quantitative trait loci (QTL) for a number of agronomic traits, including disease resistance (Lerceteau-Kohler et al. 2005), flowering habit (Sugimoto et al. 2005; Weebadde et al. 2008) and traits associated with fruit quality (Zorrilla-Fontanesi et al. 2011a). However, these studies have often employed marker types that are not easily transferrable between mapping progenies, or traits have been identified in regions where no transferrable molecular marker loci have been located. Since *F. vesca* is widely considered to be the *A* genome donor of the octoploid strawberry genome (Davis et al. 2006; Rousseau-Gueutin et al. 2009), the availability of a comprehensive genome sequence and an annotated set of anchored gene predictions for the species will greatly facilitate molecular genetics and genomics investigations in the cultivated strawberry. It will permit targeted linkage mapping, marker development and facilitate the identification of candidate genes for traits of economic importance, as has been demonstrated in the diploid *Fragaria* and in other Rosaceous species for which genome sequences exist, such as apple and peach (Velasco et al. 2010; Sargent et al. 2011; Illa et al. 2011; Vera Ruiz et al. 2011).

In this investigation we have developed a revised and updated linkage map for the cultivated strawberry using predominantly (89%) transferable sequence-characterised SSR and gene-specific markers. The map spans the expected 28 linkage groups (where one group is composed of two separate male and female maps) for an integrated F_1 linkage map of the cultivated strawberry and is estimated to almost completely cover the octoploid *Fragaria* genome. The map has highlighted regions of extensive homozygosity between linkage group homeologues, indicative of selection since the hybrid species was first produced, and a comparison with the *F. vesca* 'Hawaii 4' (FvH4) genome

sequence revealed a number of inversions between the diploid *Fragaria* chromosomes and their cultivated strawberry homeologues.

Materials and methods

Plant material

'Redgauntlet' × 'Hapil' F. × ananassa mapping population

DNA from the F_1 mapping population (RG × H) described previously by Sargent et al. (2009) was used to generate additional segregation data to increase coverage and saturation of the RG × H linkage map. The population is composed of 174 seedlings, from the cross between the two cultivated strawberry cultivars 'Redgauntlet' (female) and 'Hapil' (male). DNA was extracted using the DNeasy plant miniprep kit (Qiagen) and diluted 1:50 (~1–10 ng/μl) for use in PCR.

Microsatellite marker amplification and data generation

Selected primer pairs from the primer sets of Sargent et al. (2011), Spigler et al. (2008) and Zorrilla-Fontanesi et al. (2011b) that had not previously been mapped in RG × H were tested for heterozygosity in the parental genotypes 'Redgauntlet' and 'Hapil'. A set of 28 primer pairs flanking microsatellite loci designed by Sargent et al. (2011) from *F. vesca* 'Hawaii 4' (FvH4) genome sequence scaffolds that were not polymorphic in the diploid *Fragaria* reference map (FV × FB), but that generated heterozygous products in at least one of the two parents of the linkage map reported in this investigation, were scored in the seedlings of the RG × H progeny. Additionally, primer pairs spanning the introns of the *Fragaria* gene homologue of the cellulose bioregulator *COBRA* and a further five microsatellites developed following the methods of Sargent et al. (2009) were tested for heterozygosity in the RG × H parental genotypes.

Primer pairs, labelled on the forward primer with either 6-FAM or HEX fluorescent dyes (IDT, Belgium), that generated amplicons that were heterozygous in the parents of the RG × H progeny, were combined by product size and fluorescent dye colour into multiplexes of up to 12 primer pairs. PCR was performed using the 'Type-it' PCR mastermix (Qiagen) following the manufacturer's recommendations, in a final volume of 12.5 μl. Reactions were performed using the following PCR cycles: an initial denaturation step of 95°C for 5 min was followed by 28 cycles of 95°C for 30 s, an annealing temperature of 55°C decreasing by 0.5°C per cycle until 50°C for 90 s and 72°C for 30 s, followed by a 30-min final extension step at 60°C. PCR products were fractionated by capillary electrophoresis through a 3100 genetic analyser (Applied Biosystems). Data generated were collected and analysed using the GENESCAN and GENOTYPER (Applied Biosystems) software.

Data analysis and map construction

In many of the trace files scored, multiple dose alleles were observed segregating in the RG × H progeny. Markers deviating significantly from Mendelian single-dose segregation patterns ($P \leq 0.001$), which suggested possible multi dose segregation, were excluded from map construction since the methodology used to score trace files in this investigation meant that multiple dose segregation patterns could not be scored reliably. Alleles displaying the segregation patterns an × nn/nn × an or an × an (where a is the segregating allele and n are null or un-scored alleles), approximating to 1:1 or 3:1 Mendelian ratios, respectively, were analysed for co-segregation with other amplicons generated with the same primer pair to identify allelic pairs. Loci at which three alleles segregated for a particular marker in the conformation ab × ac (1:1:1:1) were reconstructed as a single locus.

Segregation data generated for primer pairs scored in this investigation, along with the segregation data for mapped markers presented by Sargent et al. (2009), were analysed together for map construction. Data were analysed using JOINMAP 4.0 (Kyasma, NL) 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. Following co-segregation analysis of all data, loci segregating in the conformation ab × cd (1:1:1:1) were reconstructed as single loci. Where two alleles at a single locus segregating bn × nn (1:1) and nn × cn (1:1) were identified following co-segregation analysis of all data, loci were reconstructed with the allelic conformation ab × ac (1:1:1:1), where segregation of the unscored an × an (3:1) allele could be inferred from the segregation data of the other two alleles. Those loci where only two alleles (i.e. bn × nn and an × an or nn × cn or an × an) could be scored could not be reconstructed as single-marker loci and are presented on the linkage map as separate alleles segregating at the same locus.

When all co-segregation data were analysed together, multi-point linkage analysis in some cases separated allelic pairs generated from a single primer pair and segregating in the conformation bn × nn and an × an or nn × cn and an × an on the linkage map. In all cases, alleles were separated by small genetic distances, generally less than

one centi-Morgan (cM). The raw data for these alleles were re-checked for scoring errors, then co-segregation was performed using only these markers, and loci were placed together on the linkage map using the more reliable estimated positions of the markers segregating bn × nn or nn × cn. A final segregation dataset, where all multi-allelic loci were reconstructed where possible, was re-analysed using JOINMAP 4.0 and the resultant combined maps for each linkage group were plotted using MAPCHART 2.2 for Windows (Voorrips 2002).

Locus nomenclature

Prefixes were assigned to the presented mapped marker loci according to their allelic composition. Markers consisting of alleles segregating only in the female 'Redgauntlet' parent were prefixed (a) whilst those segregating only in the male 'Hapil' parent were prefixed (b). Those markers segregating in both parents in the allelic conformation ab × ab (1:2:1), or an × an (3:1) were prefixed (c), those segregating ab × ac (1:1:1:1) were prefixed (d), and those segregating ab × cd (1:1:1:1) were prefixed (e). All loci are named according to the primer pairs from which they were amplified and include all allele sizes generated and scored using fluorescently labelled primer pairs on the 3100 Genetic Analyzer genotyping platform (Applied Biosystems). Where loci contained multiple dose or null alleles, the un-scored, 'missing' alleles are denoted (n).

Comparison of marker positions between RG × H and the *Fragaria vesca* genome sequence

All available sequence data for the markers mapped in the RG × H mapping progeny were used as queries for blasting against the *F. vesca* 'Hawaii 4' (FvH4) v1.1 pseudo-chromosomes (Shulaev et al. 2011; Sargent et al. 2011) using the batch BLAST server at the Genome Database for Rosaceae (GDR; http://www.rosaceae.org/bio/content?title=&url=/cgi-bin/gdr/gdr_blast Jung et al. 2008). A cut-off E value of e-15 was used and markers were only considered if a significant match to the FvH4 genome sequence was identified on the homologous *F. vesca* chromosome and if the match was to the genome sequence flanking both sides of the SSR sequence.

Results

Novel polymorphic FvH4 microsatellite and gene-specific primer pairs

The total number of novel primer pairs yielding heterozygous products in the parents of the RG × H progeny included 28 that were developed, but not reported, by Sargent et al. (2011), which did not amplify polymorphic products in the diploid *Fragaria* reference population (FV × FB). A further five microsatellite primer pairs developed in this investigation, following the methods of Sargent et al. (2009), yielded heterozygous products in the parents of the RG × H progeny. Additionally, the intron-spanning gene-specific primer pair for the *COBRA* gene generated heterozygous products in the parents of the RG × H progeny. The locus names of the 34 novel primer pairs, forward and reverse primer sequences, repeat motif and repeat length where applicable, and the expected product sizes in FvH4 are presented in Table 1.

Data analysis and linkage map construction

Novel mapped markers

In total, 160 *Fragaria* primer pairs, including 34 novel primer pairs reported in this investigation, generated heterozygous PCR products in the parents of the RG × H mapping population. The primer pairs generated segregating alleles that mapped to 330 loci on the RG × H genome. Of the 330 loci, 181 segregated in one parent approximating to a 1:1 Mendelian segregation ratio. Of these, 88 segregated in the female 'Redgauntlet' parent and 93 segregated in the male 'Hapil' parent, where 11 and 15, respectively, were composed of two segregating alleles, whilst 77 and 78 markers in 'Redgauntlet' and 'Hapil', respectively, comprised a single segregating allele. Of the remaining loci, 70 segregated in both parents approximating to a 3:1 Mendelian ratio, 20 segregated in both parents approximating to a 1:2:1 segregation ratio, and 59 loci segregated in both parents approximating to a 1:1:1:1 segregation ratio; 46 with the allelic configuration ab × ac or bn × cn and the remaining 13 with the conformation ab × cd (Supplementary Table 1).

RG × H map construction

Mapping data for the 330 novel loci were combined with data from 219 loci previously mapped in the RG × H population by Sargent et al. (2009) and following co-segregation analysis, a total of 549 loci coalesced into 28 linkage groups (where one group was composed of two separate male and female maps) spanning 2140.3 cM (Fig. 1). Of these; 316 segregated in one parent approximating to a 1:1 Mendelian segregation ratio, 150 in the female parent (of which 20 comprised two alleles) and 166 in the male parent (of which 27 comprised two alleles); 95 segregated in both parents approximating to a 3:1 Mendelian ratio; 28 segregated in both parents approximating to a 1:2:1 segregation ratio; and the remaining 110 loci

Table 1 The locus names, forward and reverse primer sequences, repeat motif, repeat length and the expected product sizes in FvH4 of the 34 novel primer pairs developed in this investigation

Primer pair	Forward primer	Reverse primer	Repeat motif and length	Expected product size
FvH4165	AAGCTGGGTGTTCACAGTGG	GGTCTAAAATCAGTCCGATACC	(AG)37	326
FvH4159	ACGATCTGGAGATACAACATGG	GAGGTGAAAGCAGACCTTATCC	(CT)26	328
FvH4161	AGACCTTTCATGGTGTGATGG	AAGATTGAATAGCCCGTTCC	(GA)38	180
FvH4153	ATTTTGGGCGGACTACATACC	CAAATCAAGCTACCCAGACACC	(TC)16	337
FvH4154	CAGACTCAGCAGAGTGATTTGG	CTAATGGCCCGTATCTTCTCC	(AG)9	140
FvH4178	CAGAGGGTTTGATTTGACAGC	CGTAGTACAGTACGTCATGAGTGG	(CT)28	255
FvH4167	CATTCGAACCAGAACATGTCG	GAGTCAACCACAACCTTCAACC	(CT)12	321
FvH4177	CCGAATCATTGCTCAACTACC	GAGAAAGACAGCCACTATTCAGG	(GA)26	314
FvH4176	CGGTGTAGCCTTCTTGATCG	AAGGGAAAATGCCCAAATGC	(GA)38	270
FvH4179	CTATGAGCTTTGCTTCAACACG	TGAGTTGAGAAATGAGCTGAGG	(CT)25	246
FvH4162	CTTGTTGAACAGGTTTCCAAGG	AGATCTTTTGTGTGGCTTCACC	(TC)26	288
FvH4155	GACATGGGACATGGTTTTGC	ATCCACTGATCGGAAAAGAAGG	(TA)15	328
FvH4171	GCACAGTTCCATCAGTTTTTCA	ACAATTGATCCCTTGGAAATTG	(ATC)12	208
FvH4174	GCGTTACCATGAAGAAGAAAGC	ACAGACACCAAACCTTGATACG	(AC)14	154
FvH4169	GCTACGATACAACGGAGATATGG	CATATATGGGTCACGTGAAAGG	(AG)13	220
FvH4173	GGAAATGTTACACACGGAGAGC	GGTCAAAAATGGGGTCAAAAGG	(GA)18	335
FvH4158	GGGAGCCTCAGAAAGGAACC	CTGCAGAATGCGTTCATATTCC	(AG)9	335
FvH4168	GGTACATGTCTTGGTGTCTTGG	CTCCAAATTGGTCATTCAGAGG	(AC)8	346
FvH4166	GTCTTCCCACTCATTTCTCACC	TGATGATATCCCTCACAACACC	(AT)9	232
FvH4172	TCAACGAAGATGGTAGATGAGC	CACGATTATGAACACCCAAACG	(TA)15	237
FvH4163	TCGTTCCATTGCTTATGTACCC	GAAATCCTTACCTCCCTGATGG	(CA)6	263
FvH4164	TCTCTCTCTCAATGCACAATGC	TCTTGTTTTTCCCTCTCACTGC	(GA)9	121
FvH4156	TGAATGATCAGGACAGGTATGC	CTTGGAGAGAGGTGACAATCC	(AC)12	225
FvH4170	TGCCAATCGTTGGTAAAAGA	CAAGGACACAAGGGAACTGAA	(AG)19	226
FvH4175	TGGGGGAGAGATAGATAGATGG	CGTTTCACCTACGACTGTTAGC	(AG)20	129
FvH4160	TGTGTTATTGGTCCACATAGGC	GCAGGAAGTTTTCGGCTAGG	(TC)33	291
FvH4157	TAGGTATTCCACGACAACAAGG	GATGGTTGAATCAGACAGATCG	(CT)34	298
FvH4180	GTCAACGGGACAGACTACTTCC	GCTACTTTGCTCATGTCCTTG	(TC)21	208
BFFv09-11-02	TTTTAGTAGAACACATTTTGATCG	TTAACCAATAAAACCAACCTTCG	(AT)11	178
BFFv11-05-02	ATCTTCCCATGACTTGATCAGC	CCAAGTGTCAGATTCTTTGTCC	(GA)10	216
BFFv14-29-02	AATACCAAACAGAAGCAAAATCC	TTTGAATATGCTAGCTCAAGG	(GA)27	214
BFFv16-07-01	CGATCTGGTTCTTTACCTCTGC	TTGTCCTATCACAGATGTTGTCG	(CT)41	241
BFFv16-07-02	CCGGATCTGAAATTTATTACACC	GTCGGACATATATCGACGTTGC	(CT)56	201
COBRA	TGGCACTGTAACTATCACTTGG	TATTGTTGCAGGTTGAATATCG	n/a	393

segregated in both parents approximating to a 1:1:1:1 segregation ratio, 77 with the allelic conformation ab × ac or bn × cn, and 33 with the allelic conformation ab × cd (Supplementary Table 1). At 44 loci, one allele segregating 3:1 and one allele segregating 1:1 were present, but the third allele segregating 1:1 was either a null allele or could not be scored as it segregated as a multi-dose allele at more than one locus ("Materials and methods"). The two alleles at each of these 44 loci are presented as separate alleles at the same locus on the linkage map (Fig. 1), but have been counted in the total marker numbers detailed above only as segregating in a 3:1 ratio. Of the 549 loci mapped in the RG × H progeny, 85 (15%) displayed segregation distortion; 71 (13%) at $P < 0.05$ and 14 (2%) at $P < 0.01$. The average linkage group length on the RG × H was 76.4 cM; the longest linkage group was FG6C at 110.5 cM in length, whilst the shortest was FG7D at 40.0 cM. The average whole number of loci mapped per linkage group was 19, with FG1B and FG3A containing the most markers (40) and FG5D and FG7D containing the least (10). Between linkage groups marker order was well conserved, with only small rearrangements in marker order consisting of just single markers observed between homeologous regions of the genome. A total of 33 primer pairs, 28 of which were


FG1A
FG1B
FG1C
FG1D
FG2A
FG2B
FG2C
FG2DA
FG2DB
FG3A
FG3B
FG3C
FG3D
FG4A
FG4B
FG4C
FG4D
FG5A
FG5B
FG5C
FG5D
FG6A
FG6B
FG6C
FG6D
FG7A
FG7B
FG7C
FG7D

◀ **Fig. 1** An integrated genetic linkage map of the *F.* × *ananassa* mapping progeny RG × H composed of 549 molecular markers. Prefixes and colours are given according to the allelic composition of each marker: (*a*) markers segregating only in the female 'Redgauntlet'; (*b*) markers segregating only in the male 'Hapil' parent; (*c*) markers segregating in both parents in the alleleic conformation ab × ab (1:2:1), or an × an (3:1); (*d*) markers segregating in both parents in the conformation ab × ac (1:1:1:1); (*e*) markers segregating in both parents in the conformation ab × cd (1:1:1:1). Allele sizes scored at each locus are given as suffixes to the marker names; where loci contained multiple dose or null alleles, the un-scored, 'missing' alleles are denoted (*n*). Linkage groups are named following the convention of Sargent et al. (2009), genetic distances are given in centi-Morgans (cM)

scored for the first time in this investigation, generated markers that mapped to two or more non-homeologous genomic locations on the RG × H genome. Many of the primer pairs amplified products that mapped to two distinct genomic locations (e.g. FvH4117; FG4 and FG5); however, a small number amplified products in multiple locations throughout the genetic map (e.g. FvH4074; FG1, FG2, FG4 and FG6).

Distribution of marker loci

Markers segregating in the genomes of both parents were distributed throughout the majority of the linkage groups resolved in this investigation; however, linkage groups FG1A, FG2DB, FG4B, FG6B and FG7A contained a total of six regions of in excess of 20 cM each where no markers were mapped. Additionally, not all linkage groups contained markers that segregated in both parental linkage maps: Four linkage groups were composed of only markers segregating in the female 'Redgauntlet' parental map (FG2DA) or in the male 'Hapil' parental map (FG2DB, FG3D and FG6D); and a final group (FG5D) was composed of markers segregating only in the female 'Redgauntlet' parental linkage map, except for a single marker (cFvH4115-301) segregating 3:1 in both parents. Two markers at the proximal end of linkage group FG6B were not mapped following the criteria set out in the materials and methods, and thus their positions are placed tentatively on the linkage group, denoted by (§) following the marker names. Supplementary Table 1 lists the alleles present at the 549 loci mapped in the RG × H population, detailing the linkage group to which each was mapped, the monogenic marker segregation ratios and their associated Chi-squared values.

Map coverage and estimation of genome size

Using the methods of Fishman et al. (2001), the genome size estimate for the *F.* × *ananassa* genome based on the RG × H map presented here is 2358.2 cM. The observed length of the RG × H map was 2140.3 cM and thus it is estimated to cover 91% of the *F.* × *ananassa* genome.

Comparison of RG × H marker positions to the 'FvH4' genome sequence

Of the available sequences for primer pairs mapped in the RG × H progeny, 155 gave significant matches to homologous sequences on the seven FvH4 pseudo-chromosomes, corresponding to 266 loci on the RG × H map. Marker order between the *F. vesca* genome sequence and the RG × H map was generally well conserved. The positions of the majority of markers corresponded well, with only changes in the positions of single markers between the diploid and octoploid genome and many chromosomes displaying almost complete conservation of marker order (Supplementary Figure 1). However, on two pseudochromosomes (FG1 and FG3) there was evidence of a chromosomal inversion event between the diploid and octoploid genomes delimited by the positions of numerous genetic markers on the RG × H map and conserved across the homeologous RG × H linkage groups (Fig. 2).

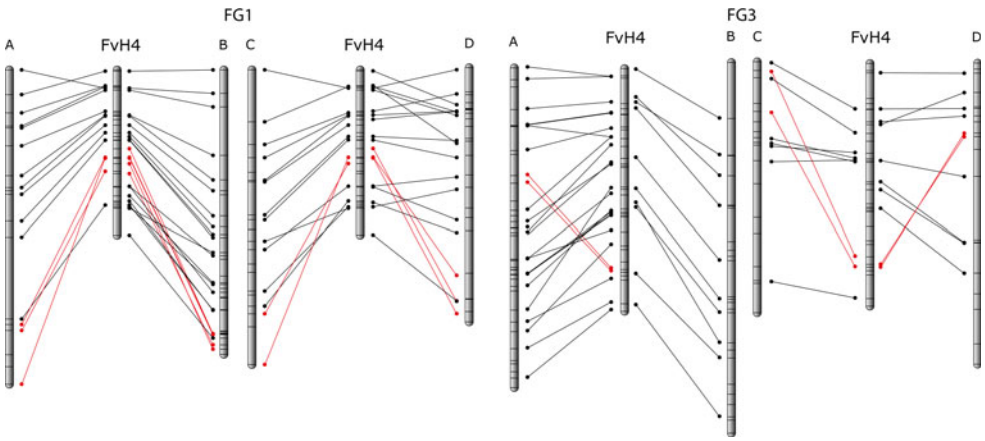

Fig. 2 A comparison of marker positions on chromosomes FC1 and FC3 of the FvH4 genome sequence and their linkage group homeologues on the RG × H linkage map displaying evidence of chromosomal inversions between the diploid and octoploid genomes

Discussion

Development of a comprehensive microsatellite marker framework for *F.* × *ananassa*

We have produced a SSR-based linkage map for the cultivated strawberry *F.* × *ananassa* spanning the 28 expected linkage groups (where one group was composed of two separate male and female maps) for an integrated parental map and covering a total of 2140.3 cM. The map presented is 1.7× the length of the SSR-based linkage map of Zorrilla-Fontanesi et al. (2011a) and almost exactly the same length (98% of the 2195 cM) of the AFLP-based linkage map of Rousseau-Gueutin et al. (2008) that was complemented with SSR markers, but which contained 32 linkage groups; four more than the number expected for an integrated map. We have added a total of 330 novel genetic loci to the map of Sargent et al. (2009); the loci were generated from 160 primer pairs, 34 of which have been mapped in *Fragaria* for the first time here to create a map containing 549 genetic marker loci, 490 of which are transferrable SSR or gene-specific marker loci composed of one to four segregating alleles. The addition of these markers has more than doubled the number of transferrable loci mapped in the RG × H progeny, significantly extending the previous map of this population reported by Sargent et al. (2009), and has defined a comprehensive integrated mapping framework for the cultivated strawberry genome based on highly polymorphic, transferrable SSR marker loci, as well as the addition of genes of known function. It covers an almost identical distance to the previous saturated AFLP map of Rousseau-Gueutin et al. (2008) and is estimated to span over 90% of the *F.* × *ananassa* genome.

Colinearity of diploid and octoploid genomes

The positions of transferrable markers on the RG × H linkage map has permitted a comparison of loci on each of the homeologous linkage groups of the *F.* × *ananassa* map and has demonstrated, as in previous investigations (Zorrilla-Fontanesi et al. 2011b; Spigler et al. 2010; Rousseau-Gueutin et al. 2008), that the subgenomes of the octoploid strawberry species are essentially collinear. Despite concluding that major chromosomal rearrangements have not been frequent in the evolution of the octoploid species, in the previous linkage maps of the cultivated strawberry (Rousseau-Gueutin et al. 2008; Zorrilla-Fontanesi et al. 2011a), intra-chromosomal inversions were observed on linkage groups FG1, FG3 and FG4. However, in each case these inversions were supported by evidence from just a single SSR marker. In this investigation, the relative saturation of many linkage groups with transferrable SSR markers permitted a more precise comparison of the diploid and octoploid genomes, revealing the same inversions on linkage groups FG1 and FG3 supported by greater numbers of SSR markers mapped to all four homeologous linkage groups of each *F. vesca* chromosome (Fig. 2). Additional support for the inversion on FG4 was also observed, but this inversion was shown on the map presented here by data from just a single SSR marker (CO816938; Supplementary Figure 1). Thus, in line with previous studies, this investigation has demonstrated that the genomes of the diploid and octoploid *Fragaria* species are highly collinear and that very little structural rearrangement has occurred since polyploidisation took place, as has been reported in other polyploid species (Liu et al. 2001; Salmon et al. 2005).

Evidence for the intra-chromosomal inversions identified was found on each of the four homeologous linkage groups, indicating that they occurred following the hybridisation of the diploid genome donors of the allo-octoploid ancestral species, but before polyploidisation occurred. This suggests that the ancestral polyploid *Fragaria* species was formed following somatic doubling of F_1 interspecific hybrids following a two-step polyploidisation process (Chen and Ni 2006). This is in contrast to the hypothesis of Rousseau-Gueutin et al. (2009) who proposed that the octoploid *Fragaria* arose from unreduced gametes in the hybridisation of two distinct tetraploid ancestral genome donors. It is also possible, however, that the observed chromosomal rearrangement occurred in the ancestral lineage of *F. vesca* and *F. bucharica*, the species from which the genetic linkage map used to anchor the *F. vesca* genome sequence was developed (Sargent et al. 2006). Future development of genetic linkage maps of other diploid *Fragaria* species would help demonstrate which is the more plausible hypothesis and would lead to an understanding of the degree of rearrangement that has occurred in the genomes of the various *Fragaria* species since they diverged from a common ancestor.

Regions of extensive homozygosity within the *F.* × *ananassa* genome

A number of previous investigations have developed genome-wide linkage maps for the cultivated strawberry (Zorrilla-Fontanesi et al. 2011a; Rousseau-Gueutin et al. 2008). In the investigation of Rousseau-Gueutin et al. (2008), 26 female and 24 male linkage groups were determined using both AFLP and SSR markers. In this case two female and one male linkage group homeologues were composed of two separate linkage group fragments each. Zorrilla-Fontanesi et al. (2011a) recovered a total of 25 female and 26 male linkage groups using AFLP and SSR markers. They reported 11 female and eight male linkage

groups homeologues were composed of more than one linkage group fragment, and many others were represented by linkage groups covering less than 15 cM.

A total of 240 microsatellite and gene-specific primer pairs amplified segregating loci in the RG × H mapping progeny; over one and a half times the number of primer pairs found to be polymorphic (153) from those tested in the study of Zorrilla-Fontanesi et al. (2011a), and more than three times the number of SSR primer pairs (79) amplifying polymorphic products from those tested in the study of Rousseau-Gueutin et al. (2008). The large number of transferrable markers used in the current investigation has allowed us to define the same regions within homeologous linkage groups and to identify distinct regions of homozygosity within the cultivated strawberry genome, some of which span entire linkage groups of the male and female maps. Despite using greater numbers of SSR markers distributed evenly throughout the *Fragaria* genome than in previous investigations, and despite recovering 28 integrated linkage groups (where one group was composed of two separate male and female maps) representing all expected cultivated strawberry linkage group homeologues, three linkage groups contained markers from only one parent (one female marker only and two male marker only groups). On a number of other groups, 'gap' regions containing no molecular markers were observed which were well saturated with markers on their equivalent homeologous linkage groups within the map, i.e. FG1A 42-62.5 cM containing 13 segregating loci on FG1B in the same genetic distance.

In the study of Spigler et al. (2010), a genetic linkage map was developed from a wild progenitor species of the cultivated strawberry, *F. virginiana*. A total of 346 polymorphic SSR primer pairs were used to generate separate linkage maps of the male and female parents containing 319 and 331 markers, respectively. However, from their report and supplementary map figure, it was not clear how many of these markers were shared jointly between the male and female maps. The linkage maps reported provided almost complete coverage of the *F. virginiana* genome, with 28 linkage groups (four sets of seven homeologues) defined for each parent (where six linkage groups were composed of two linkage group fragments, three on the female and three on the male linkage map). However, there was no evidence on the maps presented of linkage group-wide regions of homozygosity that were apparent in the cultivated strawberry linkage maps of Zorrilla-Fontanesi et al. (2011a) and Rousseau-Gueutin et al. (2008) and in the map presented in this investigation.

Strawberry breeding and selection was initiated in Europe in the early 1800s from a small number of wild and cultivated clones, and subsequently in the USA in the mid-1800s using cultivars derived from early breeding in Europe, along with a limited number of South American *F. chiloensis*, and North American *F. virginiana* genotypes. Thus modern strawberry cultivars are derived from an extremely limited germplasm base (Hancock et al. 2010), which has been demonstrated through molecular analyses of modern strawberry cultivars (Kuras and Korbin 2010). The large number of transferable markers used in the current investigation, and the relative saturation of regions of some linkage groups in relation to their homeologues where no segregating loci were mapped, suggests that genome homozygosity resulting from a narrow founding germplasm base and successive rounds of breeding and selection, rather than a paucity of molecular markers, has resulted in incomplete saturation of the RG × H and other previously published *F.* × *ananassa* linkage maps (Zorrilla-Fontanesi et al. 2011a; Rousseau-Gueutin et al. 2008).

Breeding and selection programmes for other Rosaceous crops such as peach have also been established from a relatively limited germplasm base (Dettori et al. 2001). The majority of peach linkage maps have been constructed from F_2 progenies, but as in the F_1 maps of the cultivated strawberry, the F_2 genetic linkage map of Dirlewanger et al. (2007) constructed from the peach cultivars 'Ferjalou Jalousia' and 'Fantasia' displayed high levels of homozygosity spanning the entire length of linkage group G8. Such homozygosity was not evident on the saturated interspecific *Prunus* reference map (Dirlewanger et al. 2004) which is composed of the eight expected linkage groups associated with diploid *Prunus* species. Likewise, in wheat and related species, levels of marker polymorphism on genetic linkage maps are not evenly distributed across linkage groups, with significantly lower polymorphism on the D genome of wheat, and entire linkage groups of triticale mapping populations being absent from linkage maps of that species (Chao et al. 2009; Alheit et al. 2011). The low levels of polymorphism observed in the D genome of wheat have been attributed to reduced geneflow between *A. tauschii* and *T. aestivum* after initial hybridisation events led to the formation of the hexaploid wheat species. This was subsequently followed by further a bottleneck created during the selection and subsequent breeding of modern cultivars accompanied by genetic drift causing the loss of low-frequency alleles that are in higher abundance on the D genome (Chao et al. 2009).

The data presented in this paper are insufficient to conclude that the regions of low marker density on the RG × H map are regions displaying complete homozygosity. However, the data support the assertion that alleles of the same size are present within at least two sub-genomes, but, due to the methods employed to generate segregation data in this investigation, it was not possible to assign multi-dose alleles from a single marker to individual sub-genomes. The modern cultivated strawberry genome is

derived from just a few artificial hybridisation events in the relatively recent past. Since the regions of apparent homozygosity on cultivated strawberry linkage maps are not evident on the linkage map of the wild octoploid strawberry species *F. virginiana* (Spigler et al. 2010), we suggest that fixation of a limited number of alleles during a bottleneck imposed during the creation of *F.* × *ananassa* as a species, followed by breeding and selection of modern *F.* × *ananassa* cultivars could be responsible for the regions of apparent homozygosity, or allelic homogeneity between homeologous regions of individual subgenomes on all *F.* × *ananassa* linkage maps reported to date. In order to verify this hypothesis, segregation data would need to be generated in such a way that all alleles from a single marker could be assigned to the four sub-genomes. Such a strategy is discussed below.

Single-dose marker mapping

Homeologous microsatellite marker loci segregating in the complex cultivated strawberry often carry the same allele size in different sub-genomes, leading to multi-dose marker segregation ratios. The current investigation, along with previous investigations of the genomes of octoploid *Fragaria* species (Zorrilla-Fontanesi et al. 2011a; Spigler et al. 2010; Rousseau-Gueutin et al. 2008) did not attempt to resolve segregation of multi-dose alleles. This has led to a loss of marker information, exemplified by the number of loci segregating in the allelic conformation ab × ac in the RG × H progeny, where the a allele was scored as a null allele. Previous studies of SSR markers in polyploid plant genomes have successfully developed techniques to permit the identification of multi-dose allelic configurations in polyploid species by analysing quantitative differences in peak areas between genotypes (Nybom et al. 2004; Esselink et al. 2004). Recently, such a technique was applied to the cultivated strawberry genome (van Dijk et al. 2010) demonstrating that the approach is applicable to octoploid *Fragaria* species, providing a more precise estimation of the degree of heterozygosity at each of the homeologous loci within the genome. For breeding and selection employing a MABS strategy, it is important to be able to associate specific alleles at a marker locus with phenotypic data for traits of importance for the development of superior commercial cultivars. The ability to reliably score multi-dose marker alleles in the cultivated strawberry genome would permit a greater number of alleles per primer pair to be mapped and increase the information content of mapped loci for the purposes of breeding and selection. Additionally, such an approach would be needed to verify our hypothesis that allelic fixation has occurred during the breeding and selection of modern strawberry cultivars. Multi-dose alleles were not scored in this investigation as the internal non-segregating controls required to be able to score multi-dose alleles reliably were not included in the PCRs used to generate segregation data. In future investigations, however, key markers associated with QTL for traits important to breeding could be scored using such controls to maximise the information content of such genetic loci mapped and therefore increase their utility as tools for MABS.

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