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A genetic linkage map of microsatellite, gene-specific and morphological markers in diploid *Fragaria*

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Abstract Diploid *Fragaria* provide a potential model for genomic studies in the Rosaceae. To develop a genetic linkage map of diploid *Fragaria*, we scored 78 markers (68 microsatellites, one sequence-characterised amplified region, six gene-specific markers and three morphological traits) in an interspecific F_2 population of 94 plants generated from a cross of *F.vesca* f. *semperflorens* × *F. nubicola*. Co-segregation analysis arranged 76 markers into seven discrete linkage groups covering 448 cM, with linkage group sizes ranging from 100.3 cM to 22.9 cM. Marker coverage was generally good; however some clustering of markers was observed on six of the seven linkage groups. Segregation distortion was observed at a high proportion of loci (54%), which could reflect the interspecific nature of the progeny and, in some cases, the self-incompatibility of *F. nubicola*. Such distortion may also account for some of the marker clustering observed in the map. One of the morphological markers, *pale-green leaf* (*pg*) has not previously been mapped in *Fragaria* and was located to the mid-point of linkage group VI. The transferable nature of the markers used in this study means that the map will be ideal for use as a framework for additional marker incorporation aimed at enhancing and resolving map coverage of the diploid *Fragaria* genome. The map also provides a sound basis for linkage map transfer to the cultivated octoploid strawberry.

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Introduction

The genus *Fragaria* belongs to the family Rosaceae and is currently thought to contain 20 species (Hancock 1999). Cultivated forms of some species are grown for their 'fruit', a fleshy receptacle (accessory fruit) known as the 'strawberry', which is typically juicy and sweet, often highly aromatic, and bears the true fruit ('seeds' or achenes) on its surface. Twelve of these species are diploid ($2n=2x=14$), whilst the others exhibit three levels of polyploidy. The diploid species *F. vesca* includes the 'Woodland' strawberry, *F. vesca* ssp. *vesca*, the 'Alpine' or *semperflorens* form of which is a fruit crop of minor economic importance. The natural range of *F. vesca* extends throughout the temperate regions of the northern hemisphere (Hancock 1999). Other diploid species, such as *F. nubicola*, a self-incompatible species, are present throughout Eurasia although their geographic ranges are more restricted.

Commercial success of the cultivated octoploid strawberry species *F.* × *ananassa* ($2n=8x=56$) (FAOStat Agricultural Data http://www.fao.org) has intensified interest in the development of linkage maps for the genus. Cytological data suggest that it is an auto-allopolyploid (Senanayake and Bringhurst 1967) although the extent to which segregation patterns in this species are disomic or polysomic remains to be established. Lerceteau-Köhler et al. (2003) recently provided a detailed linkage map of *F.* × *ananassa*, in which 789 amplified fragment length polymorphism (AFLP) loci were assembled into 58 linkage groups; however the use of dominant AFLP markers means that this map will be difficult to transfer between species or even populations. An attractive alternative strategy for genetically complex polyploid crops is to create an initial reference map based on transferable sequence-tagged site (STS) markers from

Table 1 Locus name, primer sequences' repeat motif and cloned fragment size for five microsatellites developed from a *Fragaria nubicola* SSR-enriched genomic DNA library together with EMBL accession numbers

Locus name	Primer sequence[a] 5′ to 3′	Motif	Cloned fragment size	EMBL accession number
EMFn002	F: ggaaccccaaataccaacttt R: aaagcctgaagttgttcaataaa	$(AC)_6$	173	AJ639620
EMFn010	F: gattaatgcccagtgatggaat R: cactgtggaagtatgtgttggg	$(TC)_{22}$	191	AJ639622
EMFn017	F: ttttcaaattgttaccccatcc R: ctaaaaatcccccaaattgtga	$(TC)_{14}$	162	AJ639623
EMFn034	F: gcctcaaagatcactcatttcc R: tcttcatctctttcaacctcaaa	$(AG)_5$	136	AJ639628
EMFn049	F: gctagcgttcaaattaagacca R: ggtatcggattgaggaaatcaa	$(AG)_{30}$	235	AJ639629

[a]F, Forward; R, reverse

diploid relatives. This approach exploits the ease with which maps can be developed and interpreted in diploid species and has been applied to many economically important polyploid crop species, including alfalfa (Diwan et al. 2000) and potato (Milbourne et al. 1998). Such diploid maps can then be used as reference points for mapping the more complex genomes of the polyploid crop species.

The diploid species *F. vesca* possesses several features that make it attractive as a potential model not only for strawberry but also for the Rosaceae. For instance, the species is characterised by a very small genome size (164 Mbp) that is comparable to the 157-Mbp size of the *Arabidopsis thaliana* genome as measured by a comparable cytometric technique (Bennett et al. 2003) and only marginally larger than the 125-Mbp *Arabidopsis* genome sequence (Akiyama et al. 2001). *F. vesca* is also amenable to genetic transformation and regeneration (El Mansouri et al. 1996; Haymes and Davis 1998; Alsheikh et al. 2002), which appears to be more difficult to achieve in other model rosaceous organisms (Ye et al. 1994). The plants themselves are small, herbaceous and comparatively rapid-cycling, with a generation time of 12–20 weeks, and so large populations can be rapidly produced for mapping using a minimum of field or glasshouse space (Battey et al. 1998). In addition, *F. vesca* can be asexually propagated via stolons, allowing clonal replicates to be used in field or glasshouse trials. Furthermore, *F. vesca* can be intercrossed with a morphologically diverse array of diploid *Fragaria* species (Bors 2000) to produce highly polymorphic interspecific hybrid populations that segregate for a range of morphological traits, including flowering time, floral and fruiting morphology and deciduousness. Such populations allow investigations of the genetic basis of such traits through the analysis of quantitative trait loci (QTL). Thus, the generation of an STS reference map for diploid *Fragaria* may have utility in broader synteny studies of the Rosaceae, a family containing economically important genera that include several fruit crops (e.g. *Malus*, *Pyrus*, *Rubus*, *Prunus*) and ornamentals (e.g. *Rosa*, *Potentilla*, *Sorbus*), and ultimately may play a role in improving our understanding of the genetic basis of polycarpic perenniality (Battey et al. 1998).

Davis and Yu (1997) were the first to produce a linkage map for *F. vesca*, although the almost exclusive use of random amplified polymorphic DNA (RAPD) analysis for that work renders their map difficult to transfer to other studies without first converting these markers to sequence-characterised amplified region (SCAR) anchor points (Paran and Michelmore 1993). There have been advances recently in the production of transferable markers for use in map-based studies of the genus. These include the development of gene-specific primers for protein-encoding genes involved in the anthocyanin biosynthesis pathway (Deng and Davis 2001) and a large number of co-dominant simple sequence repeat (SSR) markers from both the diploid (James et al. 2003; Sargent et al. 2003; Hadonou et al. 2004a, b; Cipriani, unpublished data) and octoploid (Nourse et al. 2002; Ashley et al. 2003) species. These markers have yet to be assimilated onto a reference linkage map.

We have constructed a genetic linkage map of an F_2 population derived from an interspecific cross between two diploid *Fragaria* species, *F. vesca* f. *semperflorens* (FDP815) and *F. nubicola* (FDP601), using informative STS markers developed for *Fragaria*, including five new SSR loci, and three morphological markers.

Materials and methods

Mapping population and DNA extraction

A cross was made between *Fragaria vesca* ssp. *vesca* f. *semperflorens* FDP815 (♀) and *F. nubicola* FDP601 (♂). The resultant F_1 seedlings displayed good fertility, and an F_2 progeny of 94 seedlings (population EMF001) was subsequently raised. The F_2 population segregates for three recessive morphological characters derived from parent FDP815 that are under major-gene control—non-runnering (*r*), perpetual flowering (*s*) (Brown and Wareing 1965) and a novel pale-green leaf (*pg*) phenotype. DNA was extracted from young leaves of the 94 F_2 seedlings using the DNeasy Plant Miniprep kit (Qiagen, Valencia, Calif.) according to the manufacturer's protocol. The DNA was diluted to a stock concentration of 5:200 (approximately 1.0 ng/μl) for use in PCR analyses.

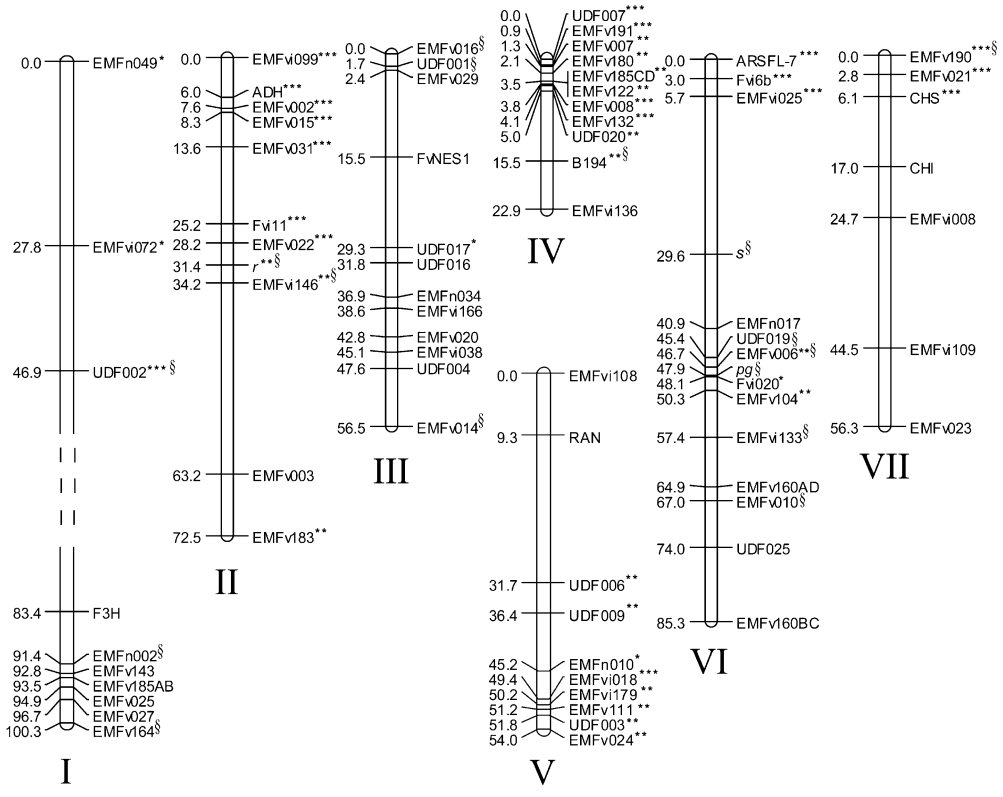

Fig. 1 Genetic linkage map constructed from an F_2 progeny from the cross of *F. vesca* f. *semperflorens* FDP815 × *F. nubicola* FDP601 showing 68 SSR, six gene-specific, one SCAR and three morphological markers. Mapping distances are given in centiMorgans (cM). The association between markers UDF002 and F3H (LOD=2.5) is shown with a *broken line* to indicate suspect linkage. Seven linkage groups (*I–VII*) follow the nomenclature of Davis and Yu (1997). 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. Dominant markers are indicated by the suffix *§*

Source and development of markers

A total of 174 PCR-based STS markers were tested on DNA from accessions FDP815 and FDP601 to assess polymorphism between the parental genotypes and probable segregation in the F_2 population. These markers comprised SSR markers (Nourse et al. 2002; James et al. 2003; Sargent et al. 2003; Ashley et al. 2003; Hadonou et al. 2004a, b), gene-specific markers (Deng and Davis 2001; A. Aharoni, personal communication) and SCAR markers (Davis et al. 1995; Haymes et al. 2000; Albani et al. 2004) that had been previously reported for *Fragaria* species along with 20 unpublished SSR markers that were retrieved from the GenBank database (Cipriani, unpublished data; GenBank accession nos. BV097098–097117). In addition, five novel SSR markers developed from a gDNA library (*F. nubicola* FDP601) enriched for SSR sequences following the protocol described by Sargent et al. (2003) also segregated in the F_2 population (Table 1).

Marker analysis

All PCRs were performed in a final reaction volume of 12.5 µl comprising 1.5 µl template DNA, 1× PCR buffer, 1.5 m*M* Mg^{2+}, 200 µ*M* dNTPs, 0.2 µ*M* each primer and 0.25 U *Taq* polymerase (Invitrogen, Carlsbad, Calif.) following the touchdown protocol described by Sargent et al. (2003) between 55°C and 50°C. PCR products were visualised and genotypes scored using one of three methods based on the size polymorphism observed between parental genotypes. Large polymorphisms (larger than 20 nucleotides) were detected by electrophoresis at 110 V for 2 h on a 1.5% (w/v) TAE agarose gel that was subsequently stained in an ethidium bromide solution (0.5 µg/ml) and visualised over UV. Smaller sized polymorphisms (smaller than 20 nucleotides) were detected either by electrophoresis on a EL500 Spreadex gel (Elchrom, Geneva) (75 V for 1 h 25 min) and staining with SYBR gold (Molecular Probes, Eugene, Ore.) for 30 min or by labelling the forward primer with either a 6-FAM, VIC, NED or PET fluorescent dye (Applied Biosystems, Foster City, Calif.) and fractionating the products by capillary electrophoresis through a 3100 genetic analyser (Applied Biosystems). Data generated by capillary electrophoresis were assembled using the GENESCAN (Applied Biosystems) software, and allele sizes were assigned with the aid of the GENOTYPER (Applied Biosystems) software.

Phenotypic markers

The F_2 seedlings were grown in an unheated glasshouse and classified phenotypically for three qualitatively segregating recessive morphological traits. They were scored as either runnering (*R*-) or non-runnering (*rr*), seasonal (*S*-) or perpetual flowering (*ss*) and green (*Pg*-) or pale-green leaf (*pgpg*) following four separate observations made at 3-month intervals.

Table 2 Co-segregation analysis of the three morphological traits (*pg*, *r*, and *s*) scored in population EMF001 with the two closest flanking markers with which co-segregation analyses could be performed (*CI* confidence interval)

Locus pairs	Observed segregation[a]	Expected segregation if loci unlinked	χ^2	Rf and (95% CI)
pg				
EMFn017	7 *aa b_* 23 *aa nn* 46 *ab b_* 0 *ab nn* 13 *bb b_* 0 *bb nn*	3:1:6:2:3:1	61*	0.072 (0.03, 0.14)
Fvi020	0 *aa b_* 23 *aa nn* 58 *ab b_* 0 *ab nn* 13 *bb b_* 0 *bb nn*	3:1:6:2:3:1	94*	0 (0, 0.02)
r				
EMFv022	0 *aa b_* 10 *aa nn* 44 *ab b_* 2 *ab nn* 37 *bb b_* 1 *bb nn*	3:1:6:2:3:1	69.78*	0.058 (0.02, 0.13)
EMFvi146	3 *aa b_* 10 *aa nn* 0 *ab b_* 0 *ab nn* 78 *bb b_* 3 *bb nn*	3:1:6:2:3:1	50.39*	0.17 (0.09, 0.26)
s				
EMFvi025	19 *aa b_* 22 *aa nn* 39 *ab b_* 6 *ab nn* 7 *bb b_* 1 *bb nn*	3:1:6:2:3:1	17.73*	0.26 (0.17, 0.36)
EMFn017	11 *aa b_* 19 *aa nn* 41 *ab b_* 5 *ab nn* 10 *bb b_* 3 *bb nn*	3:1:6:2:3:1	24.03*	0.23 (0.15, 0.34)

*Significant at P=0.0001
[a]*nn* in observed segregation ratios indicates recessive phenotype

Data analysis and map construction

Chi-squared tests of goodness-of-fit to an expected segregation ratio of 1:2:1 for co-dominant markers and 3:1 for dominant markers were carried out for all molecular and morphological markers. Linkage analysis was performed and the map constructed using JOINMAP 3.0 (Van Ooijen and Voorrips 2001) applying the Kosambi mapping function. Linkage groups were constructed and marker order determined using a minimum LOD score threshold of 3.0, a recombination fraction (Rf) threshold of 0.35, a ripple value of 1.0, a jump threshold of 3.0 and a triplet threshold of 5.0. Co-segregation of locus pairs (where presented in the text) was tested with the contingency chi-squared test for linkage (Mather 1951) using LINKEM v1.2 for MS-DOS (Vowden et al. 1995). Rf, standard error (SE) and LOD scores are given.

Markers shared by this study and an unpublished work on an intraspecific *F.vesca* mapping population (Hadonou and Sargent, unpublished data) were carefully compared to match the prior numbering of the seven *F. vesca* linkage groups of Davis and Yu (1997) to those defined by the current investigation.

Results

Marker analysis in population EMF001

Of the 174 molecular markers tested (including five novel *F. nubicola* SSRs), 75 exhibited polymorphisms between the parental genotypes and so were used to develop a map from the F_2 population. These markers comprised 68 SSR loci, six gene-specific markers and a single SCAR marker. Fifty-eight of the molecular markers scored consisted of co-dominant band mobility polymorphisms, but 17 segregated as present (dominant):absent (recessive) polymorphisms. The latter included 14 SSRs that had been originally developed from *F. vesca* genomic libraries and amplified only in FDP815 (*F. vesca*) and three SSRs (EMFn002, EMFvi088 and EMFvi146) developed from *F. nubicola* and *F. viridis* libraries that amplified only in *F. nubicola* (FDP601).

The majority of the 75 primer pairs that revealed polymorphisms generated alleles that mapped to a single genetic locus. However, two primers (EMFv160 and EMFv185) each revealed segregation at two loci. These loci were denoted EMFv160AD and EMFv160BC, and EMFv185AB and EMFv185CD, according to the relative gel mobilities of the PCR products when subjected to electrophoresis. Significant deviation from the expected 3:1 or 1:2:1 ratios was detected at 54% (42 out of 78) of the polymorphic loci screened. Additional information regarding the markers mapped in this investigation, including monogenic marker segregation ratios and the associated χ^2-values, are available on the Genomic Database for Rosaceae http://www.genome.clemson.edu/gdr. 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 in Fig. 1.

Co-segregation analysis and map construction

Following linkage analysis, 76 of the 78 markers coalesced into seven discrete linkage groups (Fig. 1), covering a total map distance of 448 cM. In linkage group I (LGI), the significance of the association between UDF002 and F3H was less than the threshold LOD=3.0 [r=0.32 (SE=0.058), LOD=2.5]; thus these markers are tentatively included on LGI but shown linked with a broken line to represent the suspect association. With this reservation, LGI was the longest linkage group found. The linkage groups ranged in size from 100.3 cM (ten loci) for LGI to 22.9 cM (11 loci) for LGIV, with a mean length of 64 cM and 11 markers per linkage group. The distribution of markers was far from even, with zones of marker clustering apparent on six of the linkage groups. Significantly skewed segregation ratios were distributed across all linkage groups and could account for some of the observed clustering of loci.

The six gene-specific markers were located to five linkage groups (LGI, II, III, V and VII), whilst the three morphological markers were positioned on two (LGII and VI). The markers used to match the prior numbering of the seven *F. vesca* linkage groups of Davis and Yu (1997) to those defined by the current investigation were as follows: LGII, RAPDs B102C and PO20A, SSRs EMFv002 and EMFv022; LGIII, OPZ14CD and EMFv014; LGV, OPZ04CD and EMFv024; LGVII, OPX18AB and EMFv006. LGI and LGVII were assigned using the map positions of the gene-specific markers F3H (LGI) and CHI (LGVII) reported by Deng and Davis (2001), and the remaining linkage group was deduced to be LGIV.

Map position of morphological characters

The morphological characters *s* and *pg* segregated in accordance with the expected ratio of 3:1 and were linked to each other at a map distance of 18 cM on LGVI [Rf=0.15 (SE=0.041), LOD=7.76]. The other morphological marker, non-runnering (*r*), deviated significantly from the 3:1 expected segregation ratio (81:13) and mapped to the mid-point of LGII. The co-segregation data for the three morphological markers and the closest flanking molecular markers with which co-segregation analyses could be performed are given in Table 2. The *pg* locus (a morphological marker not previously mapped) was flanked by SSRs Fvi20 (0.2 cM) and EMFv006 (1.2 cM). The closest flanking markers to the *r* locus were EMFv022 (3.2 cM) and EMFvi146 (2.8 cM), whilst the closest markers to *s* were EMFvi025 (23.9 cM) and EMFn017 (11.3 cM).

Discussion

Our genetic linkage map derived from an interspecific *F. vesca* × *F. nubicola* F_2 progeny includes seven discrete linkage groups, corresponding to the chromosome base number of the genus (*x*=7) and reports the map position of a novel mutant *Fragaria* gene, *pg*. Genome coverage of the 76 markers/448 cM is nearly identical to the 80 markers/445 cM coverage of the prior RAPD-derived *F. vesca* map (Davis and Yu 1997) and the map has a comparable total length to those of other genera within the Rosaceae of a similar genome size, such as *Prunus* (Joobeur et al. 1998). However, two segregating markers remain unlinked, suggesting that at least two parts of the genome have not been covered by this first reference map for the genus, and it is probable, given the absence of any proportionately shorter chromosomes in the reported karyotypes of diploid *Fragaria* species (Iwatsubo and Naruhashi 1989), that these markers lie beyond the ends of one or more of the linkage groups reported here upon which marker coverage is still low, such as LGIV (22.9 cM).

The map uses transferable SSR and gene-specific markers and therefore creates a framework which can form the basis for future mapping studies within the genus as well as, in the case of the gene-specific markers, providing the potential for synteny studies with other Rosaceous genera. This facility may also accelerate map-based research in the commercial octoploid strawberry and provide opportunities for marker-assisted selection and eventually for the positional cloning of genes of potential economic importance from both the diploid and octoploid *Fragaria* species. It will also facilitate synteny studies between the octoploid and diploid genomes by direct comparisons between homeologous anchor markers. Such endeavours would permit the transfer of information on gene position and genome organisation to the cultivated strawberry from surrogate studies in the diploid *Fragaria*. Possible examples include the identification of the genes involved in fruit colour (Deng and Davis 2001) and the seasonal flowering locus (Albani et al. 2004).

To make a linkage map with maximum generic utility, we used an interspecific cross in order to increase the number of segregating loci over an intraspecific *F. vesca* cross, which would likely be far less polymorphic. However, a consequence of such a wide cross can be skewed segregation ratios, as reported for other species (Paterson et al. 1988; Kianian and Quiros 1992; Brummer et al. 1993; Kiss et al. 1993; Bradshaw et al. 1995; Joobeur et al. 1998), which can arise from incomplete homologous pairing, or selection, between the parental species.

Whilst the level of distorted segregation ratios observed in this investigation was high (54%), it is broadly comparable with the results of other studies involving wide interspecific crosses (Zamir and Tadmor 1986). It is also in line with the findings of Davis and Yu (1997) who reported similar levels of segregation distortion (47%) in a genetically narrower intraspecific diploid *Fragaria* cross. In that study, Davis and Yu (1997) reported that all skewed loci were biased towards an excess of alleles from *F. vesca* f. *semperflorens*, the maternal grandparent. They speculated that the *semperflorens* cytoplasm may have provided a gametic or zygotic cellular environment that favoured the transmission of *semperflorens* alleles. In our study, the majority of skewed loci (80%) were biased towards an excess of alleles from the paternal crossing parent (FDP601). Clearly the explanation presented by Davis and Yu (1997) for segregation distortion in their linkage map cannot account for the observed pattern of segregation distortion revealed here, and alternative causes must be considered. These might include irregular segregation at meiosis and the self-incompatible nature of *F. nubicola*, which may have contributed to the segregation distortion of markers on the chromosome carrying the self-incompatibility locus (*S*), as has been reported for *Prunus* (Joobeur et al. 1998).

Despite the segregation distortion, the majority of linkage associations between markers in this study were strongly supported. All but one association (indicated by a broken line in Fig. 1) were supported by LOD scores in excess of the minimum of 3.0 as laid out in the initial criteria for map construction.

Our map shows four distinct regions of marker clustering—at the ends of linkage groups LGI, LGIV and LGV and at the mid-point of LGVI (Fig. 1). A similar degree of SSR clustering is apparent on the *Malus* × *domestica* map of Liebhard et al. (2002), particularly on linkage groups F2b, F5, F10, F13 and F14 in that study. Such clustering could be the result of suppressed recombination at the telomeric and centromeric regions of the chromosomes. Interestingly, however, the SSRs mapped in a recent interspecific map of *Pyrus* (Yamamoto et al. 2002) were more evenly distributed throughout the linkage groups and clustering was not apparent.

Application of common RAPD, SSR and gene-specific markers in related mapping studies (Deng and Davis 2001; Davis et al. unpublished data), has allowed determination of linkage group correspondence with the published map of Davis and Yu (1997), upon which basis linkage group numbers were assigned to the present map (Fig. 1).

The alcohol dehydrogenase (*ADH*) and non-runnering (*r*) loci were reported by Davis and Yu (1997) to be linked at a distance of approximately 40 cM. However, the pairwise Rf calculated by LINKEM v1.2 was 0.25 (SE=0.055, LOD=4.75) (Davis, unpublished), which corresponds well to the Rf calculated for the linkage between the two loci in this investigation, 0.27 (SE=0.053, LOD=2.45).

In the study by Deng and Davis (2001), using a number of different mapping populations, *CHS* was placed on LGIII (linked to B102B at 29.0 cM) whilst *CHI* located to LGVII (linked to PO20C at 21.5 cM) (Deng and Davis 2001). In our investigation, these two markers were found to be closely linked [r=0.13 (SE=0.027), LOD=17.85], and both were assigned to LGVII. This apparent incongruity could be the consequence of a reciprocal translocation event since the divergence of *F. vesca* and *F. nubicola* and therefore pseudolinkage between the loci in the interspecific progeny (Jáuregui et al. 2001). An alternative explanation derives from the nature of the populations used in their study. The primary aim of the Deng and Davis (2001) investigation was to establish correspondence between one of the anthocyanin genes and the yellow fruit colour mutation in *F. vesca* Yellow Wonder. To this end, small mapping populations containing just 40 individuals were used to establish linkage between the codominant STS markers and dominant RAPD markers originally mapped by Davis and Yu (1997). Such co-segregation analyses can be poorly informative, especially in small populations, and this may have led to either the *CHI* or *CHS* marker being erroneously associated with one of the RAPD markers.

Conclusions

We have presented a molecular map for the genus *Fragaria* that has been constructed entirely from robust, transferable, PCR-based markers, appropriate for future studies. The reproducible nature of the markers means that we have produced a framework onto which additional markers can be incorporated to enhance and resolve the genetic distance of our map and genome coverage of the diploid *Fragaria* genome and, thus, we have provided the framework for the development of transferable linkage maps of the cultivated strawberry.

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