

# Comparative Mapping Reveals Autosomal Origin of Sex Chromosome in Octoploid *Fragaria virginiana*

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## Abstract

Recent evolution of separate sexes in flowering plants provides unparalleled opportunities for understanding the early stages of sex chromosome evolution, including their origin from autosomes. Moreover, the transition from combined to separate sexes can be associated with speciation via polyploidization in angiosperms, suggesting that genome doubling/merger may facilitate sterility mutations required for sex chromosome formation. To gain insight into the origin of sex chromosomes in a polyploid plant, we doubled the simple sequence repeat (SSR) density and increased genome coverage in a genetic map of octoploid *Fragaria virginiana*, a species purported to have a “proto-sex” chromosome, where limited recombination occurs between 2 linked “loci” carrying the male- and female-sterility mutations. Incorporation of almost 3 times the number of SSR markers into the current map facilitated complete characterization of the *F. virginiana* proto-sex chromosome, revealing its largely autosomal nature and the location of the sex-determining region toward the distal end. Furthermore, extensive synteny between our genetic map and a map involving diploid hermaphroditic congeners allowed assignment of linkage groups to homeologous groups, identification of the proto-sex chromosome’s autosomal homoeolog, and detection of a putative rearrangement near the sex-determining region. Fine mapping and additional comparative work will shed light on the intriguing possibility that rearrangements during polyploidization were involved in the evolution of sex chromosomes in *Fragaria*.

**Key words:** *allopolyploidy, dioecy, Fragaria, male sterility, sex chromosome evolution*

Sex chromosomes evolve from ordinary autosomes (Ohno 1967; Bull 1983). Initial steps in this transition involve a mutation that confers male sterility followed by a mutation at a second locus that causes female sterility (Charlesworth B and Charlesworth D 1978). The linkage of these 2 loci sets the stage for later steps in sex chromosome evolution that include suppressed recombination in the sex region, accumulation of sexually antagonistic genes, and finally, degeneration that leads to heteromorphism (reviewed in Charlesworth 2008). When the male-sterility mutation is recessive, this can lead to male heterogamety in an XY system; although rarer, when the male-sterility mutation is dominant, it can lead to female heterogamety in a ZW system. These evolutionary steps are thought to be so fundamental that they have been similarly traversed by organisms as diverse as animals, plants, and fungi (Fraser and Heitman 2005).

In most animal taxa, however, sex chromosomes are ancient (e.g., human ~300 million years old [MYO]) and

heteromorphic, making it impossible to detect the very earliest stages of the process, in part because degeneration erases the signature of earlier events (Fraser and Heitman 2005). In contrast, sex chromosomes in flowering plants are much younger (e.g., <20 MYO: Bergero et al. 2008; Yu, Navajas-Pérez, et al. 2008), and a great diversity of sexual systems can exist within a genus, providing the opportunity to study closely related species that differ in sexual system. In fact, genera that contain both hermaphroditic species and those with separate sexes, that is, either dioecy (females, males) or intermediate states such as subdioecy (hermaphrodites, females, and males), can possibly provide the ultimate portal into the initial transition of autosomes to sex chromosomes. Taken together, these features indicate that select angiosperm genera can be influential systems for understanding the very first events in sex chromosome evolution.

A common mode of speciation in plants is via whole genome duplication or merger, that is, polyploidy (reviewed

in Ramsey and Schemske 1998; Otto 2007). This is of particular interest in the context of sex chromosome evolution because there is a positive association between polyploidy and dioecy in angiosperms (reviewed in Miller and Venable 2000, but see Obbard et al. 2006; Volz and Renner 2008). This pattern is especially strong in lineages where the hermaphrodite diploid progenitors possess gametophytic self-incompatibility (Miller and Venable 2000; Yeung et al. 2005), suggesting that loss of self-incompatibility following polyploidization may be an important facilitator of the evolution of dioecy (Jennings 1976; Baker 1984; Miller and Venable 2000). In addition, male or female sterility is a common consequence of interspecific hybridization as a result of chromosomal rearrangements, cytonuclear or epistatic gene interactions (e.g., Comai et al. 2000; reviewed in Rieseberg and Willis 2007; Long et al. 2008), or interlocus incompatibilities (Sweigart et al. 2006). Thus, allopolyploids could be predisposed to the evolution of dioecy by virtue of the high incidence of duplications or deletions leading to sterility, that is, loss of function, mutations that, under certain ecological and genetic conditions, can confer a selective advantage (Charlesworth B and Charlesworth D 1978). Moreover, the genomic restructuring that has been seen to follow both hybridization and genome doubling (reviewed in Soltis et al. 2003; Doyle et al. 2008) could produce translocation events between autosomes that may also contribute to sex chromosome evolution (e.g., Charlesworth D and Charlesworth B 1980; Watson et al. 1991; Carvalho 2002). Regardless of the underlying mechanisms, the association between dioecy and polyploidy exists in several plant taxa (reviewed in Table 1 of Miller and Venable 2000), making the question of how sex chromosomes evolve in polyploid plants a particularly important, yet unexplored one.

Comparative genomics has provided answers to the questions of homology between sex chromosomes among related sexually dimorphic species of both plants (e.g., white campion [Mrackova et al. 2008] and papaya [Yu, Navajas-Pérez, et al. 2008]) and animals (e.g., stickleback fishes [Ross et al. 2009] and *Drosophila* [Flores et al. 2008]) and of common ancestry between X and Y (e.g., Yamato et al. 2007). Far fewer studies, however, have used comparative analysis between closely related sexually dimorphic and hermaphroditic species to identify the putative autosomal homoeolog(s) of the sex chromosomes (Filatov 2005). This latter comparison is crucial if we are to unravel the incipient stages of sex chromosome evolution, that is, before major genomic changes have occurred. Moreover, no comparative analysis has involved species that differed in ploidy level, which will be particularly compelling given the propensity for speciation by polyploidization in plants and the positive association between polyploidy and dioecy.

*Fragaria* is a genus that provides an exceptional opportunity to elucidate the mechanisms leading to the formation of sex chromosomes in polyploid plants because of the existence of multiple ploidy levels, a diversity of sexual systems including hermaphroditism and complete

dioecy, and an association between polyploidy and sexual dimorphism. *Fragaria virginiana* is an allopolyploid ( $2n = 8x = 56$ ) species with a subdioecious sexual system. A first-generation genetic map of *F. virginiana* (Spigler et al. 2008) revealed that sexual phenotype is determined by at least 2 linked gene regions, or “loci,” one controlling female function and the other male function, with limited recombination between them, leading to the prediction that this species possesses the earliest form of a sex chromosome, that is, a proto-sex chromosome (Moore 2008; Spigler et al. 2008). However, incomplete coverage in the early generation map precluded complete characterization of this chromosome, thereby restricting our insight into its origin and the genetic processes that led to its formation. Therefore in this study, we present a second-generation simple sequence repeat (SSR)-based genetic map with double the marker density and increased genome coverage. The map is now comprehensive enough to 1) allow full characterization of the proto-sex chromosome and other linkage groups (LGs) so as to assign them to one of the expected 7 homeologous groups (HG) and 2) conduct a comparative analysis using this octoploid map and a published map based on 2 hermaphroditic diploid congeners, one of which (*F. vesca*) shares a recent common ancestor with *F. virginiana* (Rousseau-Gueutin et al. 2009; Njuguna W, Bassil N, Cronn R, Liston A, unpublished data). As a result, we were able to identify the autosomal diploid homoeolog of the octoploid proto-sex chromosome and evaluate the level of macrosynteny between these chromosomes in the context of a genome-wide comparison between the 2 ploidy levels. In doing so, we shed first light on the genetic macrostructure of a sex chromosome in an octoploid plant and begin to elucidate whether genome rearrangements might be involved in sex chromosome formation in this polyploid.

## Materials and Methods

### Species Description

*Fragaria virginiana* (Rosaceae), the Virginian wild strawberry, is a perennial herb that is native to eastern North America (Staudt 1989). Cytological and genetic evidence suggests that *F. virginiana*, like other octoploid strawberries, exhibits disomic inheritance (Ashley et al. 2003; Lerceteanu-Köhler et al. 2003; Rousseau-Gueutin et al. 2008), has a genomic structure of AAA'A'BBB'B' (Bringhurst 1990), and has a relatively recent origin (~0.20 MYO; Njuguna W, Bassil N, Cronn R, Liston A, unpublished data). Although the number and identity of diploid contributors to the octoploid genome has long been debated (see discussion in Foltá and Davis 2006), there is support from several sources for a *F. vesca*-like contributor of the AA genome (Senanayake and Bringhurst 1967; Rousseau-Gueutin et al. 2009), and a recent phylogeny based on chloroplast genomes indicates that *F. virginiana* shares a common maternal contributor with *F. mandshurica* (Njuguna W, Bassil N, Cronn R, Liston A, unpublished data). All diploid congeners of *F. virginiana* are

hermaphroditic and either self-compatible (*F. vesca* and *F. iinumae*) or self-incompatible (*F. viridis* and *F. mandshurica*) (Staudt 1989; Rousseau-Gueutin et al. 2009), although there is an anecdotal report that some populations of subspecies *F. vesca bracteata* have male-sterile individuals (Ahmadi and Bringhurst 1991).

*Fragaria virginiana* is 1 of 6 sexually polymorphic polyploid members in the genus (Staudt 1989). It has a subdioecious sexual system as populations can contain varying frequencies of 3 sexual phenotypes: females, hermaphrodites, and males (Ashman et al. 1999). In addition, neuter individuals have been observed occasionally in wild populations (Valleau 1923; Ashman T-L, personal observation). Sex phenotype in *F. virginiana* is determined by at least 2 linked loci (or gene regions), with sterility alleles at each (male sterility dominant to fertility and female sterility recessive to fertility) (Spigler et al. 2008). Limited recombination between these loci can explain the variation in sexual phenotypes observed and reflects both female and hermaphrodite heterogamety when all 3 (or 4, including neuters) sexual morphs are present in a population.

### Construction of an Enhanced Linkage Map for Octoploid Wild Strawberry

The *F. virginiana* mapping population ( $N = 184$  progeny) is an  $F_1$  generation resulting from a cross between a female (Y33b2) and a hermaphrodite (O477) and has been described in detail elsewhere (Spigler et al. 2008).

The genetic map was built entirely from polymerase chain reaction (PCR)-based markers, mostly SSR markers. The current map includes markers from published SSR primers derived from multiple *Fragaria* species and blackberry (*Rubus* L.) (Davis and Yu 1997; James et al. 2003; Sargent et al. 2003, 2007; Cipriani and Testolin 2004; Hadonou et al. 2004; Gil-Ariza et al. 2006; Monfort et al. 2006; Lewers et al. 2008) and 174 newly developed, previously unpublished SSRs in addition to 172 primers that amplified polymorphic products used in the first-generation map (Spigler et al. 2008). Approximately half of the newly developed SSRs were developed from publicly available strawberry sequences downloaded from NCBI database for expressed sequence tags as described in Spigler et al. (2008) (Supplementary Table 1A) and half were developed from pooled genomic DNA from the map parents by Genetic Identification Services (Chatsworth, CA) (Supplementary Table 1B). For use in parental screens and genetic mapping, the 5' ends of the forward primers were modified with the addition of a fluorescently labeled M13 sequence (5'-TGTA AAC-GACGGCCAGT-3') (Schuelke 2000). The 5' ends of the reverse primers were modified by the addition of a short sequence (GT, GTT, or GTTT) to facilitate adenylation for more uniform product sizes (Brownstein et al. 1996).

In addition to SSRs, male function (pollen production) and female function (fruit set) were characterized for each of the progeny in the mapping population. To generate accurate assessments of these phenotypic traits, 6 clones were produced from each progeny and each clone was

scored. For the purposes of mapping sex function qualitatively, individuals were scored as male sterile if they did not produce pollen, otherwise they were male fertile, and scored as female fertile if they set 5% or more of their flowers into fruits, otherwise they were female sterile. Readers should refer to Spigler et al. (2008) for more detail. Preliminary mapping of female fertility quantitatively confirms the location of a major quantitative trait locus adjacent to the location of male function and nowhere else (Spigler RB, Lewers KS, Ashman T-L, unpublished data), further substantiating the existence of a proto-sex chromosome.

Because it is not known a priori which PCR products from a given primer pair cosegregate at a locus, all (1729) PCR products evaluated for map construction were treated initially as single-dose markers (Wu et al. 1992), and their fit according to expected Mendelian segregation ratios of either 1:1, if present in only one parent, or 3:1, if present in both parents, was evaluated as in Spigler et al. (2008). Readers should refer to Wu et al. (1992) for greater detail on using the single-dose restriction fragment method for mapping in polyploids. To identify possible cosegregating alleles of a codominant marker, we first mapped products from each primer pair in JoinMap 4.0 (Van Ooijen 2006). PCR products from a given primer pair were deemed to be allelic at a locus if they mapped to the same location and were in repulsion. For PCR products that were linked and in repulsion but did not map to the exact same position, we checked the raw data to evaluate possible genotypes. If at least 90% of the progeny scores suggested that the 2 PCR products were allelic (95% for loci involving a 3:1 marker), then we considered them as a single, codominant marker. In these cases, genotypes that did not fit the allelic model were almost always cases of the absence of both PCR products, which we interpreted to be due to allele dropout, specifically PCR errors or mutations at the primer site (Bonin et al. 2004). If less than 90% of the data suggested cosegregation, the data were left as is and the products were mapped separately. In cases in which products from the same primer pair were linked closely in coupling, suggesting possible tandem duplications, the raw data were rechecked to confirm scores. During this entire process, a few individuals ( $N = 5$ ) were found to have an excess of genotypes inconsistent with Mendelian segregation given the hypothesis of cosegregating alleles of a codominant marker; these individuals were removed from the data set.

### Construction of Individual Parent Maps

We used a pseudo-backcross strategy to create separate parent maps (Grattapaglia and Sederoff 1994) of this octoploid strawberry. Individual parent maps were constructed separately with JoinMap using the respective parental 1:1 markers, shared 3:1 markers, and codominant markers (i.e., segregation types  $ab \times ab$  or  $ab \times ac$ , where the first 2 letters in each segregation type represent the genotype of the maternal parent, the latter 2 letters represent the genotype for the paternal parent, and different letters represent unique alleles from a given marker) (Supplementary Table 2). Individuals missing data for >25% of marker

data were excluded from the data set, and markers missing data for >25% of individuals were excluded for initial group formation in JoinMap. The additional removal of individuals with missing data resulted in a total of 174 individuals included in the final mapping set. Initial groups were selected at  $6 \leq \text{logarithm of the odds (LOD)} \leq 9$ . Most groups remained stable at LOD scores greater than 6. In the few cases where groups branched into distinct clusters at successively greater LOD scores, however, we used LOD scores >6 to avoid spurious groupings. We subsequently added additional, ungrouped markers to established LGs using “strongest cross link” values given in JoinMap, using a threshold  $\text{LOD} \geq 5$ . Marker order and map distance were determined using the Kosambi mapping function, minimum LOD threshold of 3.0, recombination threshold of 0.35, and jump threshold of 3.0. For 4 LGs (I.D-p, V.D-m, V.D-p, VI.C-p; Supplementary Figure 1), the less strict default parameters (1.0, 0.40, 5.0, respectively) were used to improve goodness-of-fit statistics associated with marker positions (V.D-m, V.D-p) or to facilitate mapping of markers useful for HG assignment (I.D-p, VI.C-p) (see below). For each map where a third round of mapping was required, we chose to use the third round because a major goal was to identify HGs, and this would allow the maximum number of markers to join the map. To control for possible errors in mapping order in this round, we selectively removed markers with poor goodness-of-fit statistics. In almost all cases, the third round mapping order was identical to the first round. We calculated genetic map lengths as in Fishman et al. (2001) and estimated genome coverage using the formula of Lange and Boehnke (1982). We generated graphic maps in MapChart 2.1 (Voorrips 2002).

#### Identification of Homeologous and Homologous Groups in *F. virginiana*

To identify HGs, we grouped LGs based on SSR commonality. LGs were identified as homeologous to one another if they shared at least 2 SSRs. Both parental maps were used such that if a maternal LG had markers derived from at least 2 primers shared in common with a paternal LG already placed in a given HG but not with other maternal LGs, it was nonetheless considered to belong to that HG. A few LGs could not be placed in an HG using these criteria. Therefore, we tentatively placed these in an HG if they shared at least one SSR with an LG in that HG or if they had an SSR in common with the corresponding homeologous LG from the diploid *Fragaria* map (see below).

Once LGs were placed in HGs, we identified homologous LGs between the parent maps by comparing overall SSR similarity and order and/or shared PCR products fitting a 3:1 segregation ratio from a given primer pair. Where such assignments were not clear, an integrated map was created in JoinMap using all markers (maternal and paternal) from the HG in question. We were able to assign LGs to homologous groups using these methods for all but 2 pairs of LGs for which an integrated map could not be constructed.

#### Comparative Analysis

To identify the autosomal diploid homeolog of the octoploid proto-sex chromosome and to evaluate the level of macrosynteny between the 2 ploidy levels, we compared our enhanced genetic map to a published map derived from an interspecific cross between 2 hermaphroditic diploid congeners (*F. vesca* and *F. nubicola*) (map data kindly provided by D. Sargent) (Sargent et al. 2006, 2008) (but see Rousseau-Gueutin et al. 2008 concerning whether *F. nubicola* was instead *F. bucharica*). We only used SSRs in the diploid map that were shared with our octoploid *F. virginiana* map (Supplementary Figure 1). By assessing the distribution of shared SSRs between the 2 maps, we identified all 7 HGs in the octoploid map. Each LG in the octoploid map is named with a Roman numeral (I–VII) according to its respective diploid homeolog and a letter (A–D) for reference (Supplementary Figure 1).

## Results and Discussion

### A Comprehensive Genetic Map of Octoploid *F. virginiana*

Our map was constructed from 829 PCR-based markers, mostly SSRs, that conformed to our Mendelian segregation ratio criteria (i.e.,  $P \geq 0.0001$ ) plus the 2 sexual function traits (Supplementary Table 2). The resultant maternal and paternal *F. virginiana* maps comprised 33 and 32 LGs, respectively, compared with the 28 haploid number of chromosomes expected (Supplementary Figure 1). Through assignment to HGs (see below) and examining marker complementarities among LGs, however, we were able to identify probable associations between 5 pairs of individual LGs (III.B-m and 1-m; V.D-m and 3-m; VI.D-m and 4-m; VI.A-p and 2-p; and VI.D-p and 3-p), and thus ultimately resolved the equivalent of 30 LGs in both maps. The maternal and paternal maps comprised 319 and 331 markers, respectively (Table 1, Supplementary Table 2). Markers shared between the 2 parental maps included 10 codominant markers (i.e.,  $ab \times ab$  and  $ab \times ac$  segregation types) and 24 single-dose markers (i.e.,  $an \times an$ ). Map lengths, marker density, and genome coverage were similar and high for both parental maps (Table 1). In fact, both maps have near complete genome coverage (90% within 10 cM of a marker in both maps), although one should be cautious in interpreting such estimates as they can be subject to several sources of error (see discussion in Fishman et al. 2001). Moreover, we were able to identify probable homologs between the parent maps for all but 2 pairs of LGs (Supplementary Figure 1). Additional mapping statistics are given in Table 1.

The 28 (haploid) chromosomes of octoploid *F. virginiana* can be assembled into 7 groups of 4 homeologous chromosomes. We were able to assign all but one LG in each parental map to a HG (Supplementary Figure 1), based first on shared SSRs among LGs within the octoploid map and then through shared SSRs with the diploid *Fragaria* map. Consequently, each HG contains an LG corresponding to

**Table 1** Descriptive statistics for separate maternal and paternal *Fragaria virginiana* maps presented in Supplementary Figure 1

	Maternal map	Paternal map
Total marker number <sup>a</sup>	319	331
Number of LGs	33 (30) <sup>b</sup>	32 (30) <sup>b</sup>
Average LG length (cM)	67.1	73.4
Range of LG lengths (cM) <sup>c</sup>	49.9, 70.1, 86.0	55.9, 73.9, 89.2
Average marker interval (cM)	7.74	7.86
Range of marker intervals (cM)	3.78, 6.56, 10.86	3.81, 6.38, 10.62
Average number markers per LG	10	10
Range of markers per LG	6, 10, 13	8, 10, 13
Total map length (cM)	2213.79	2349.70
Estimated genome length	2724.66	2852.64
Genome coverage (percent within 10 cM)	0.90	0.90

<sup>a</sup> Total marker number includes 10 codominant markers (i.e., ab × ab and ab × ac) and 24 shared 3:1 markers (i.e., an × an, where “n” is a null allele). Markers that are allelic but that could not be scored as codominant within each map (e.g., ab × an) were counted as a single “locus” or marker.

<sup>b</sup> The number of LGs in each map can be reduced to 30 based on putative associations between pairs of LGs (see Supplementary Figure 1).

<sup>c</sup> Ranges are presented as the lower quartile, median, and upper quartile values.

a chromosome from each diploid contributor genome (named A–D, Supplementary Figure 1). Pairs of LGs within each HG shared an average of 3 SSRs and as many as 10 with one another. Identification of the diploid homoeolog to each HG was determined unequivocally as a result of high levels of macrosynteny between the diploid and octoploid maps (Supplementary Figure 1). HGs shared as many as 18 SSRs (as in HG VI, “HG-VI”) with their respective diploid homoeolog, and all HGs shared at least 9 SSRs with their respective diploid homoeolog. Individually, LGs in each of the octoploid HGs had as many as 9 SSRs in common with the corresponding diploid homoeolog and two-thirds of the octoploid LGs shared 3 or more SSRs with the corresponding diploid homoeolog.

### Autosomal Homoeolog of the Proto-sex Chromosome Identified

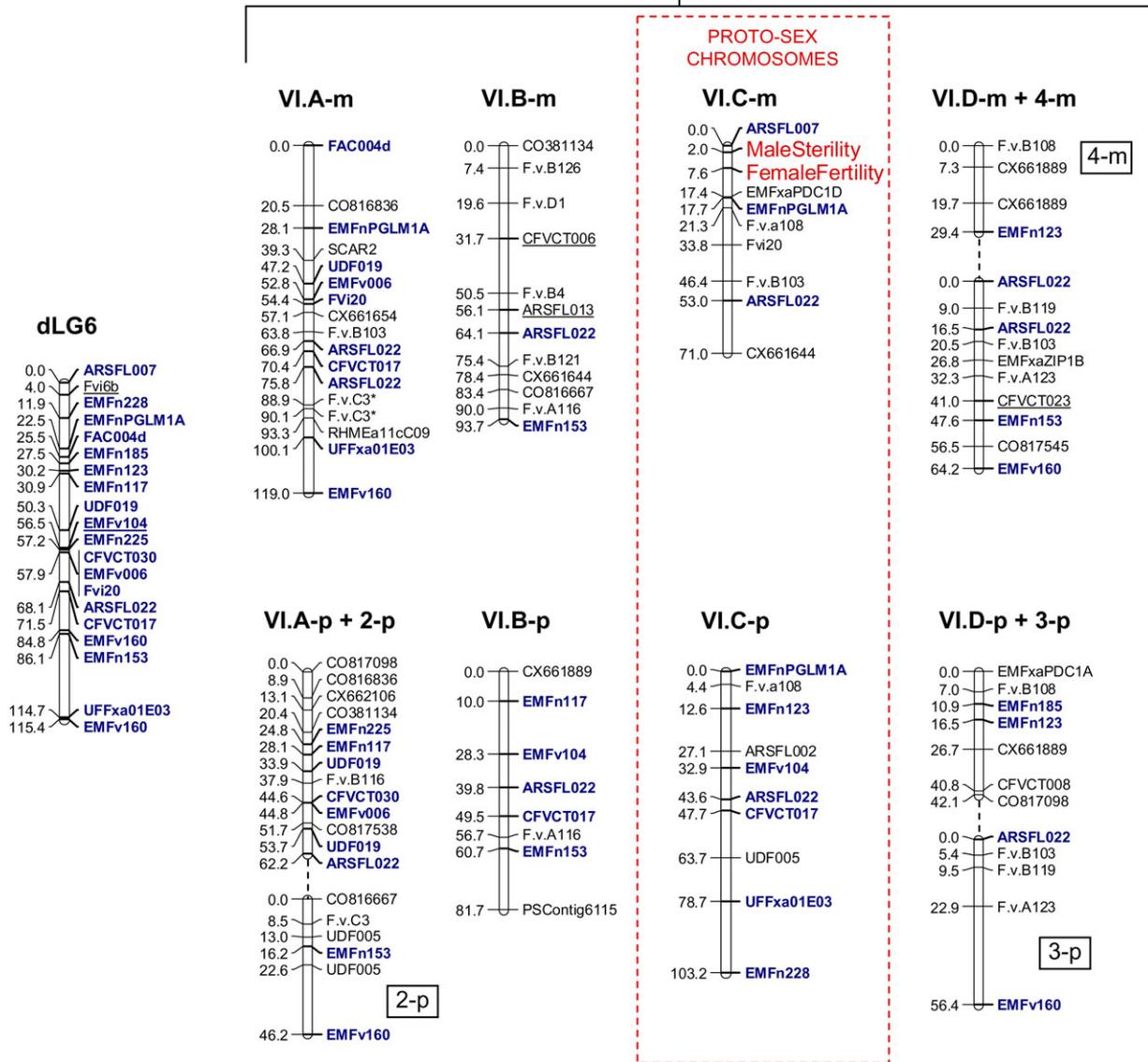
Extensive macrosynteny between our genetic map of octoploid *F. virginiana* and a published diploid map (Figure 1, Supplementary Figure 1) allowed unequivocal determination of a single diploid autosomal homoeolog to the proto-sex chromosome of *F. virginiana*. Specifically, 9 SSRs are shared between diploid linkage group (dLG) 6 and the proto-sex chromosome homologs (LGVI.C-m,p; Figure 1). The finding of synteny with a single chromosome in the map of diploid hermaphroditic congeners marks the first evidence of the autosomal origin of a sex chromosome in a polyploid species and clearly indicates that sex chromosomes were a single pair of autosomes prior to the acquisition of sex-determining loci.

The level of coverage in the current genetic map allowed for more complete characterization of the sex-determining

chromosome than previously possible. Prior to this we had only identified linkage of the 2 sex traits and 1 SSR (Spigler et al. 2008). The linkage map of the proto-sex chromosome in the map presented here, however, appears to be complete or nearly so relative to its diploid homoeolog, based on shared markers at both ends of the LGs (Figure 1). The current octoploid map reveals that the putative sex-determining loci in *F. virginiana* map toward the tip of LGVI.C. Coincidentally, this region also houses the self-incompatibility locus in a diploid *Fragaria* (Sargent DS personal communication), and it is noteworthy that recombination suppression between mating-type loci involved in self-incompatibility is favored by natural selection (Takebayashi et al. 2004) just as it is in sex-determining regions. Moreover, the sex-determining region is similarly located at the tip in *Populus* (proximal “subtelomeric” region, Yin et al. 2008), threespine stickleback (distal end, Peichel et al. 2004), and guppy (distal end, Tripathi et al. 2009), with recombination suppression in the corresponding region for all 3 species, highlighting the potential importance of proximity to the telomere. This association might occur if this region is predisposed to lower recombination rates, similar to regions near the centromere, where the sex-determining region of papaya is found (Yu et al. 2007). Yet that there is still some recombination between the sex-determining loci in *F. virginiana* further underscores the autosomal origin of the proto-sex chromosome, and the largely autosomal nature is also clearly evident from shared SSRs between the maternal and paternal homologs of the proto-sex chromosome (Figure 1, VI.C-m,p), and more importantly, shared and/or codominant SSR PCR products (EMFnPGLM1A; F.v.a108).

One possible explanation for the similarity between the proto-sex chromosome and its diploid homoeolog may be the presumably young age of the sex chromosome, and accordingly, its largely autosomal nature. This conclusion is supported not only by the young age of the species (~0.20 MYO; Njuguna W, Bassil N, Cronn R, Liston A, unpublished data) but also by the fact that there is still limited recombination between the 2 putative sex-determining loci (Spigler et al. 2008; Figure 1, Supplementary Figure 1). Thus we may expect rearrangements like inversions that lead to recombination suppression to become fixed later as a consequence of selection against the production of recombinant individuals (i.e., neuters and hermaphrodites). Alternatively, fine-scale genetic mapping and/or physical mapping may reveal rearrangements not visible at our current marker density given the small sex-determining region. Indeed, in other species with young sex chromosomes and very small sex-determining regions, such as papaya (8 Mbp, Yu et al. 2007) and threespine stickleback (estimated 10 Mbp, Ross and Peichel 2008), bacterial artificial chromosome sequencing was required to detect rearrangements (Ross and Peichel 2008; Yu, Hou, et al. 2008; Yu, Navajas-Pérez, et al. 2008). As the *Fragaria* phylogeny becomes more resolved (Potter et al. 2000; Rousseau-Gueutin et al. 2009) and species-specific markers are developed, we will be able to identify the contributor

## Homeologous Group VI



**Figure 1.** Grayscale: Parental linkage maps of octoploid *F. virginiana* LGs (VI.A–VI.D) in the HG containing the proto-sex chromosome as compared with a diploid *Fragaria* homoeolog (dLG6). Octoploid LGs are named according to the HG (VI) and a letter (A–D), which was assigned first in the maternal map according to largest to smallest LG (in cM) and then accordingly to the corresponding homologs in the paternal map. Additional LGs in the octoploid map beyond the 4 expected are named using Arabic numerals. Lower case “m” and “p” are given to denote LGs in the maternal and paternal maps, respectively. SSRs on the octoploid HG-VI LGs that are shared with the diploid homoeolog are highlighted in bold. Underlined marker names on dLG6 are involved in putative genome rearrangements in the octoploid (see text and Table 2A). Phenotypic trait markers representing the putative determining sex loci are indicated in enlarged font on the proto-sex chromosome (VI.C). The entire genetic map of *F. virginiana* is presented in Supplementary Figure 1.

genomes of allopolyploid *F. virginiana* and thus not only recognize gene transfers between genomes that may not be apparent from macrosynteny alone (e.g., via fluorescence in situ hybridisation or genomic in situ hybridisation, Zhao et al. 1998; Lim et al. 2004) but also pinpoint the ancestral genomic contributor(s) of the proto-sex chromosome.

Yet despite such overwhelming synteny among the LGs of HG-VI and dLG6, there are incongruities suggesting

genome rearrangements in the octoploid relative to the diploid (Table 2A, Figure 1, Supplementary Figure 1). Interestingly, LGs of HG-VI were more likely to contain SSRs found in dLGs other than the expected diploid homoeolog than any other HG. Most of these likely represent duplications followed by translocations, given that the SSRs involved are found in multiple locations and/or produced more PCR products than mapped. Other notable

**Table 2** SSRs and locations indicating genomic rearrangements in octoploid *Fragaria* based on comparisons (A) between the diploid and octoploid maps and (B) within the octoploid map

SSR	Location in diploid map <sup>a</sup>		Location in octoploid map <sup>b</sup>	
	LG	LGs	HGs	LGs
A. Between the diploid and octoploid maps				
ARSFL013	1		<b>VI</b>	<b>VI.B-m</b>
CFVCT006	4		I, II, <b>VI</b>	I.A-m; II.B-p; <b>VI.B-m</b>
EMFv122	4		V	V.C-m,p
EMFn181	5		III	III.C-m,p
EMFv104	<b>6</b>		III, <b>VI</b>	III.C-p; <b>VI.B-p</b> ; <b>VI.C-p</b>
Fvi6b	<b>6</b>		I	I.B-m,p
CFVCT023	7		<b>VI</b> , VII	<b>VI.D-m</b> ; VII.B-m,p
B. Within the octoploid map				
F.v.B121	n/a		I, IV, <b>VI</b> , VII	I.A-p; IV.D-p; <b>VI.B-m</b> ; VII.A-m,p
F.v.C103	n/a		I, VII	I.A-p; I.B-m,p; I.D-p; VII.A-p
F.v.C113	n/a		I, VII	I.D-p; VII.A-p
F.v.C3	n/a		I, III, IV, <b>VI</b>	I.A-m; I.C-m,p; III.A-m; III.C-m,p; III.D-m,p; IV.B-m,p; <b>VI.A-m</b> ; 1-m; <b>2-p</b>
F.v.D1	n/a		I, <b>VI</b>	I.A-m; <b>VI.B-m</b>

Items in bold highlight involvement of diploid LG6 and HG-VI, which contains the proto-sex chromosome. n/a, not applicable.

<sup>a</sup> Diploid map adapted from Sargent et al. (2006, 2008); see Supplementary Figure 1.

<sup>b</sup> Refer to Supplementary Figure 1 for map and details.

rearrangements involve SSRs found in dLG6 that are either found in additional locations in the octoploid map or missing from HG-VI (Table 2A). Most interestingly, Fvi6b, which is found at the tip of LG6 in the diploid, did not map to any of the HG-VI LGs in the octoploid and instead mapped to the tip of a single homologous pair (LGI.B-m,p) in HG-I (Table 2A, Supplementary Figure 1). This is particularly noteworthy because this marker is close to ARSFL007 in the diploid map (Figure 1, Supplementary Figure 1) and ARSFL007 maps close to the sex-determining region in the octoploid (Figure 1, Supplementary Figure 1; Spigler et al. 2008). In a map of a hermaphrodite cultivated octoploid strawberry, *F. × ananassa* (Sargent et al. 2009), Fvi6b products mapped to LGs in both HG-I and HG-VI, leading the author of that work to propose that LG1 and LG6 have a historical association involving a duplication event. In our map, however, all products from Fvi6b mapped to what is presumably a single locus on LG1.B. This is not entirely surprising given that Fvi6b was initially developed as a locus-specific marker in *F. virginiana* (Ashley et al. 2003), and together, these lines of evidence suggest that a nonreciprocal translocation or duplication and translocation followed by a deletion of Fvi6b may have occurred during the evolution of *F. virginiana*. The 2 copies found in *F. × ananassa* may be attributable to its hybrid origin, that is, its genome reflects chromosomes from both *F. virginiana* and *F. chiloensis*, which are themselves both derived from an *F. vesca*-like ancestor (Rousseau-Gueutin et al. 2009) that may have contained one copy of Fvi6b on LG6.

Based on these lines of evidence alone, we cannot conclude that a deletion or other rearrangement, possibly as a result of polyploidization, is directly related to sex determination or proto-sex chromosome formation in

octoploid *F. virginiana*, but even small genomic changes have been shown to be linked to loss of normal sexual function and sex chromosome evolution in other species. For example, a single gene duplication and insertion event is involved in sex determination in medaka (Nanda et al. 2002), and losses of DNA due to nonreciprocal homeologous gene transfer in *Brassica* polyploids led to reduced seed or pollen fertility (Gaeta et al. 2007). Likewise, significant changes in other important traits, such as flowering time in *Brassica*, have similarly involved transpositions as a result of polyploidy (Pires et al. 2004). If the deletion event of Fvi6b is related to loss of male or female sex function in *F. virginiana*, then we might expect a different mechanism or even a different LG to be involved in determining sex phenotype in *F. chiloensis*, which presumably still retains Fvi6b on at least one chromosome in its HG-VI. Comparative mapping is underway to test this hypothesis.

### Genome-Wide Rearrangements in the Octoploid

Comparisons between the entire *F. virginiana* octoploid map and the diploid map can further inform on whether the rearrangements that involve HG-VI are simply representative of those seen across the entire genome or are unusual (e.g., either because this group is predisposed to rearrangements or because they have been selectively maintained) and may provide clues into the evolution of the proto-sex chromosome in this polyploid plant. Across the entire map we found 2 additional SSRs (EMFn181 and EMFv122) that differed in location between the diploid and octoploid maps (Table 2A; Supplementary Figure 1). Interestingly, translocations of EMFn181 are also evident in the cultivated congener *F. × ananassa* on multiple LGs

(Sargent et al. 2009). In *F. virginiana*, however, a non-reciprocal translocation is indicated because all 4 PCR products from EMFn181 mapped to a single locus in our octoploid map.

In addition to rearrangements inferred from deviation from synteny with the diploid map (Table 2A), we saw evidence of duplications across the octoploid genome based on markers specific to our map (Table 2B, Supplementary Figure 1). Associations of loci with more than one HG may not be uncommon, and in fact, may be found in blocks suggesting evidence of chromosome segment duplications as was found in cotton (Brubaker et al. 1999). Most of these inter-HG markers in our map occurred individually, and thus could represent individual duplications and transpositions, however, for a few LGs, these markers are found near one another (e.g., I.A-m, VI.B-m, and VII.A-p).

It is worth mentioning that none or few of the types of the rearrangements or duplications observed in our map of a wild octoploid strawberry were detected in 2 published maps of the cultivated octoploid strawberry (*F. × ananassa*: Rousseau-Gueutin et al. 2008; Sargent et al. 2009). This may reflect the consequences of selective breeding in the cultivar (including a severe reduction in genetic diversity) and/or its hybrid origin, or alternatively, differences in the number of SSRs between the maps, given that our map has greater than twice the number of SSRs per LG compared with those maps. Continued development of these octoploid genome maps will ultimately allow interesting comparisons, particularly between the sex-determining region in *F. virginiana* and the homoeologous region in the cultivar, where hybridization and artificial selection has presumably led to the loss of sterility alleles and fixation for both fertility alleles, that is, hermaphroditism.

Ultimately, our findings reveal few dramatic genomic changes following polyploidization, consistent with those found in the cultivated strawberry maps (Rousseau-Gueutin et al. 2008; Sargent et al. 2009) and cotton (Liu et al. 2001) but in contrast to others (*Brassica* [e.g., Song et al. 1995] and wheat [e.g., Feldman et al. 1997]). These differences among taxa may reflect different methodological approaches, or more interestingly, different routes to polyploidization.

### Implications and Perspectives

Genome restructuring during polyploidization can lead to male and/or female sterility (e.g., Comai et al. 2000; Gaeta et al. 2007), suggesting a possible mechanism by which polyploidy can facilitate the evolution of sex chromosomes and a possible additional explanation for the observed positive association between dioecy and polyploidy in flowering plants (Miller and Venable 2000). The only way to evaluate this hypothesis is to compare the genomes of sexually dimorphic polyploids that have sex chromosomes with their hermaphroditic diploid congeners, and the key first step in this approach is the development of detailed genetic maps. This study represents the first to compare

a map of a sexually dimorphic species with that of hermaphroditic congeners and, more to the point, the first to involve such species that also differ in ploidy level.

Our results add to a growing body of evidence supporting the hypothesis that the earliest stages of sex chromosome evolution involve the acquisition of 2 sterility mutations on a single autosome that otherwise retains its autosomal nature (Ohno 1967; Bull 1983). In addition, despite remarkable macrosynteny among homeologs within the octoploid and between the octoploid and diploid homoeologs, our map reveals minor genomic changes including duplications and translocations throughout the genome, including at least one on the proto-sex chromosome. The intriguing possibility that these rearrangements played a role in modifying sexual phenotype (i.e., male sterility or female sterility) in *F. virginiana* demands further attention given the large phenotypic effects that gene loss, duplications, and translocations can have (e.g., Nanda et al. 2002; Osborn et al. 2003; Gaeta et al. 2007). Such work is underway and will aid in elucidating the mechanism underlying a potentially important pathway to sex chromosomes in plants.

More generally, closer examination of the proto-sex chromosome in *F. virginiana* will allow us to capture the initial stages of sex chromosome evolution, that is, possibly even before complete recombination suppression of the sex-determining region has been achieved. Comparative work underway involves other sexually dimorphic species in *Fragaria*, including dioecious *F. chiloensis*, to gain further insight into the evolution of sex chromosomes in this genus. This work can inform on whether the sex chromosomes of these species evolved from the same set of autosomes, as in papaya species (Yu, Navajas-Pérez, et al. 2008), for example, thereby providing the full evolutionary sequence within a single genus, or whether there were independent origins, as was found in other species (Takehana et al. 2007; Mrackova et al. 2008; Ross et al. 2009). In fact, the 2 scenarios might not be mutually exclusive in *Fragaria* given that polyploid events in 2 separate lineages (Rousseau-Gueutin et al. 2009; Njuguna W, Bassil N, Cronn R, Liston A, unpublished data) have led to clades containing both hermaphroditic diploid and sexually dimorphic polyploid species. It is also worth considering the possibility that the first sterility mutation, that is, male sterility, came from a common diploid ancestor related to the modern subspecies *F. vesca bracteata*, the sole diploid purported to have male sterility (Ahmadi and Bringhurst 1991), in which case the second sterility mutation, female sterility, which is reported not to exist in *F. vesca bracteata* (Ahmadi and Bringhurst 1991) but is required to produce a sex chromosome, might have been facilitated by polyploidy. Again, comparative analysis is needed to determine whether there is a common genomic signature with respect to the dominant male sterility mutation but not for female sterility across these 3 *Fragaria* species. Taken together, the diversity of ploidy levels and sexual dimorphism, presence of female heterogamety (Valleau 1923; Bringhurst 1990; Spigler et al.

2008), and young age makes *Fragaria* an extraordinary model genus for understanding the evolution of sex chromosomes.

## Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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