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ORIGINAL ARTICLE

Sex-determining chromosomes and sexual dimorphism: insights from genetic mapping of sex expression in a natural hybrid *Fragaria × ananassa* subsp. *cuneifolia*

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We studied the natural hybrid (*Fragaria × ananassa* subsp. *cuneifolia*) between two sexually dimorphic octoploid strawberry species (*Fragaria virginiana* and *Fragaria chiloensis*) to gain insight into the dynamics of sex chromosomes and the genesis of sexual dimorphism. Male sterility is dominant in both the parental species and thus will be inherited maternally, but the chromosome that houses the sex-determining region differs. Thus, we asked whether (1) the cytotypic composition of hybrid populations represents one or both maternal species, (2) the sex-determining chromosome of the hybrid reflects the location of male sterility within the maternal donor species and (3) crosses from the hybrid species show less sexual dimorphism than the parental species. We found that *F. × ananassa* subsp. *cuneifolia* populations consisted of both parental cytotypes but one predominated within each population. Genetic linkage mapping of two crosses showed dominance of male sterility similar to the parental species, however, the map location of male sterility reflected the maternal donor in one cross, but not the other. Moreover, female function mapped to a single region in the first cross, but to two regions in the second cross. Aside from components of female function (fruit set and seed set), other traits that have been found to be significantly sexually dimorphic in the pure species were either not dimorphic or were dimorphic in the opposite direction to the parental species. These results suggest that hybrids experience some disruption of dimorphism in secondary sexual traits, as well as novel location and number of quantitative trait locus (QTL) affecting sex function.

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INTRODUCTION

Natural hybrids can form when two species with incomplete reproductive isolation come into secondary contact (Rieseberg and Carney, 1998). Once viewed as 'evolutionary noise' (Wagner, 1970), hybrid zones are now being viewed as natural laboratories for ecological and evolutionary studies in speciation and diversification (for example, Rieseberg and Wendel, 1993; Sweigart, 2009) as they provide insight into the prevailing direction of gene flow, gene introgression and adaptation (for example, Nolte *et al.*, 2009; Wallace *et al.*, 2011). It also has been noted that natural hybrid zones are an underexploited source of information on reproductive isolation and mating (reviewed in Rieseberg and Blackman, 2010), early sex chromosome evolution (Veltsos *et al.*, 2008) and the genetics of sexual dimorphism (Coyne *et al.*, 2008). Hybrid zones may be where new sterility alleles are expressed (reviewed in Rieseberg and Blackman, 2010) or old ones rearranged (for example, Petit *et al.*, 2010). Such novelty could lead to the evolution and spread of new sex-determining chromosomes (Veltsos *et al.*, 2008; Pannell and Pujol, 2009). Moreover, because sexual dimorphism (or sex limitation) that evolved separately in the two species can breakdown in the hybrids (Parker and Partridge, 1998), the pattern of dissolution or re-expression of male traits in females can provide information on the origin and type of genetic control underlying sexual dimorphism (Coyne *et al.*, 2008).

Although there are a handful of well-studied plant hybrid zones that involve gender dimorphic (that is, dioecious (males and females), or gynodioecious (females and hermaphrodites)) parental species; for example, Buggs and Pannell, 2007; Minder *et al.*, 2007; Lexer *et al.*, 2010; Wallace *et al.*, 2011), there are few where we also have explicit knowledge of the location of sex-determining genes in the parental species. In fact, we have very few studies that compare genetic maps of sex determination in hybrids with their parent species (but see, Paolucci *et al.*, 2010; Macaya-Sanz *et al.*, 2011), or the level of sexual dimorphism in the hybrid to that of its parental species. Yet, it is in these systems where we will be most readily able to address questions of novel locations of sex-determining genes and of the effect of hybridization on sexual dimorphism.

As a first step in addressing these gaps, we investigated the level of population admixture and the location of the sex-determining region in two populations of *Fragaria × ananassa* subsp. *cuneifolia*, a natural hybrid of two octoploid species, *Fragaria chiloensis* and *Fragaria virginiana*. These are the same two species that were cultivated in Europe in the 1700s, and hybridized to produce the cultivated strawberry *F. × ananassa* subsp. *ananassa* (Darrow, 1966). Both parental species show gender dimorphism and sexual dimorphism in secondary traits (Ashman, 2003, 2005; Ashman *et al.*, 2011; Spigler *et al.*, 2011) but they differ in the chromosome

that houses the sex-determining region (Goldberg *et al.*, 2010). *F. chiloensis* is predominantly dioecious (Hancock and Bringhurst, 1979b) and recent mapping studies have revealed that sex is determined by a dominant sterility allele (*A*) at the male function 'locus' and recessive sterility allele (*g*) at the female function 'locus' and that these 'loci' colocalize on linkage group (LG) VI.A (Goldberg *et al.*, 2010). *F. virginiana* is subdioecious, and sex expression is also controlled by a dominant male-sterility allele and a recessive female-sterility allele but here linkage between the two sex function 'loci' is less complete (that is, recombination can lead to hermaphrodites and neuters), and these major sex-determining loci are on LG VI.C (Spigler *et al.*, 2010). The dominance and different chromosomal locations of male sterility means that, barring rearrangements, the location of sex-determining genes in the hybrid should reflect that of the maternal donor species. That is, when *F. chiloensis* is the donor we expect male sterility to map to LG VI.A but when *F. virginiana* is the maternal donor it will map to VI.C. One can use species-specific plastome markers to identify the maternal donor of individuals and the populations they derive from (Minder *et al.*, 2007; Arnold *et al.*, 2010) to facilitate testing this hypothesis. In addition, as several of the sexually dimorphic secondary traits have been shown to be controlled, in part, by loci colocalizing with the sex-determining region (Ashman *et al.*, 2011; Spigler *et al.*, 2011), we predicted that dimorphism in these traits would be less in the hybrid than found in the pure species crosses.

Specifically, we sought to determine whether (1) two populations of *F.* × *ananassa* subsp. *cuneifolia* reflect a single parental species or a mixture of the two parental species as maternal donors; (2) the sex-determining chromosome of the hybrid reflects the location of male sterility within the maternal donor species; and (3) crosses from the hybrid species show less sexual dimorphism than published reports for the parental species.

MATERIALS AND METHODS
Species description and study populations
F. × *ananassa* subsp. *cuneifolia* is a perennial stoloniferous herb that inhabits edges of pastures, pine forests, roadways and the interfaces of woods and back dunes (Hancock and Bringhurst, 1979a; Staudt, 1999). It is morphologically intermediate to the parental species (Salamone *et al.*, in preparation; Staudt, 1999) and currently exists in a narrow hybrid zone that stretches from southern British Columbia to northern California (Staudt, 1999). Although the two progenitor species last shared a common ancestor 400 000–1 770 000 years ago based on the 95% highest posterior density of a Bayesian dating analysis (Njuguna *et al.*, 2012), the timing of secondary contact is unknown. Staudt (1989) speculated that hybridization occurred after glaciation in the Fraser River valley of British Columbia receded. Evidence from diagnostic plastome single-nucleotide polymorphisms indicates that either species can act as the maternal donor (Salamone *et al.*, in preparation). Cytological and genetic evidence also suggests that both wild octoploid species and the cultivated hybrid *F.* × *ananassa* subsp. *ananassa* have a genomic structure of AAA′A′BBB′B′ (Bringhurst, 1990), and exhibit disomic inheritance ($2n = 8$ $x = 56$) (Ashley *et al.*, 2003; Lerceteau-Köhler *et al.*, 2003; Rousseau-Gueutin *et al.*, 2008). *F.* × *ananassa* *cuneifolia* is subdioecious with three sexual morphs (hermaphrodites, males and females) co-occurring within a population (Ashman personal observation; Staudt, 1999), similar to one of its progenitor species *F. virginiana* (Ashman and Hitchens, 2000).

We collected 32 *F.* × *ananassa* subsp. *cuneifolia* plants along transects through each of two populations in Benton County, OR, USA (Wren (WREN) (44.5878 N, 123.4272 W, 133 m) and Mary's Peak (MP) (44.5044 N, 123.55 W, 1203 m)). All three sexual phenotypes were observed in these populations and females represented ∼50% in each population (Wren: 47%, MP: 53%). Plants were grown in 200 ml pots filled with a 2:1 mixture of Fafard #4 (Conrad Fafard) and sand in the greenhouse at the University of Pittsburgh. Plants received fertilizer and protection from pests as needed.

Creation and cultivation of F. × ananassa subsp. cuneifolia mapping populations
To map sex determination, we created two mapping populations by crossing plants derived from WREN and MP populations. In MP12 × WREN2 cross a MP female (67% fruit set) was pollinated with pollen from a WREN individual that was male fertile but set no fruit. In the WREN7 × MP10 cross, a WREN female with 96% fruit set was pollinated with pollen from a MP hermaphrodite (32% fruit set). We pollinated female parents with pollen collected from the male parents during February through April 2010 and planted 144 seeds from each cross in May 2010. Seeds were planted in 72-well trays with a custom germination mix (Sunshine germination mix: Fafard #4: sand), and exposed to 14-h days and 15 °C/20 °C night/day temperatures in a growth chamber. Germination was high (both crosses ∼95%), and after 2 months of growth seedlings were transplanted into 200 ml pots filled with a 2:1 mixture of Fafard #4 and sand. At this time, we also produced two clones of each parent. All plants were exposed to 12 °C/22 °C night/day temperatures and 12-h day light for 4 months before a 2-month dark treatment at 4 °C. Growth conditions during flowering were 11-h day light at 12 °C/18 °C night/day temperatures. We hand-pollinated each flower on all plants three times per week with outcross pollen to ensure full potential fruit and seed set. During the entire course of study, all plants received seven beads of granular nutricote 13:13:13 N:P:K fertilizer (Chisso-Ashai fertilizer, Sun Gro Horticulture, Bellevue, WA, USA) and were protected from pests as needed.

Sex expression and phenotype data
Sex expression was scored on each plant at least twice during flowering. As in previous studies (Goldberg *et al.*, 2010; Spigler *et al.*, 2010), we scored male function qualitatively based on the presence or absence of pollen production and this was assessed in at least two flowers per plant. Individuals with yellow anthers visibly releasing pollen were scored as 'male-fertile', whereas plants that produced white vestigial stamens and whose anther sacs lacked pollen were scored as 'male-sterile'. Female function was quantitatively estimated as the percentage of flowers that produced fruit ('fruit set'). To be consistent with the previous qualitative mapping of female function in *F. virginiana* (Spigler *et al.*, 2008) and *F. chiloensis* (Goldberg *et al.*, 2010), we considered plants with ⩾5% fruit set as 'female fertile' and those with ⩽5% fruit set as 'female sterile'. We scored sex expression on all the flowering plants in the mapping populations and present the data for the representative subset that were genotyped. In addition, we scored several phenotypic traits that have been shown to be sexually dimorphic in *F. virginiana* and/or *F. chiloensis* (proportion seed set, anther number per flower, flowers per plant, leaf number per plant and runner number per plant) following the protocols described in Spigler *et al.* (2011).

For each F1 mapping population, we determined whether there was sexual dimorphism between male-sterile and male-fertile morphs using *t*-tests. To facilitate comparisons with published indices of sexual dimorphism in parental species (Ashman *et al.*, 2011; Spigler *et al.*, 2011), we calculated a sexual dimorphism index following McDaniel (2005) as $|(x_{MS} - x_{MF})|/[(s.e._{MS} + s.e._{MF})/2]$, where x and s.e. are the mean and standard error, respectively, for each trait for male-sterile (MS) and male-fertile (MF) morphs.

DNA extraction
DNA was extracted from 10–15 mg of silica-dried young leaf tissue from the 32 plants per population for cytotyping and from progeny (85 from MP12 × WREN2, 90 from WREN7 × MP10) and two replicates of the parents of the two crosses for genetic mapping. We used a CTAB extraction protocol (Doyle and Doyle, 1987) modified to accommodate a 96-well high-throughput format. DNA was quantified using a Spectromax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) and diluted to 0.3 ng μl^{-1} with deionized sterile water for PCR reactions.

Cytotyping
To distinguish the parental donor of *F.* × *ananassa* subsp. *cuneifolia* individuals, we screened each individual for two single-nucleotide polymorphisms that differentiate *F. virginiana* and *F. chiloensis* chloroplast genomes (Njuguna *et al.*, 2012; Salamone *et al.*, in preparation). Single-nucleotide polymorphisms

within the intron of *pet*D and an exon of *ndh*F were screened in 32 plants per population using the dCAPs technique (Neff *et al.*, 1998).

Specifically, we amplified the two chloroplast regions *pet*D (primers petDintron-77875F (5′-GGATAGGCTGGTTCGTTTGA-3′), petDintron-78409R (5′-GCTCGAGCATGAATCAACAG-3′)) and *ndh*F (ndhF-113272F (5′-AAAATCCCCGACACGATTAG-3′), ndhF-113799R (5′-ACCGTTCATTC-CACTTCCAG-3′)). PCR reactions included $1 \times$ PCR buffer ($10 \times$ buffer with MgCl$_2$, Qiagen, Valencia, CA, USA), 100 μM of each dNTP, 0.5 μM of each forward and reverse primer, 1.5 units of *Taq* polymerase and 1 μl of genomic DNA in a 20 μl reaction. PCR amplification began with a hot start of 95 °C for 2 min to activate the *Taq* polymerase (New England Biolabs, Beverly, MA, USA) followed by 94 °C for a 45-s denaturation step, followed by 35 cycles of: (1) 45-s denaturation at 94 °C; (2) 30-s annealing at 51 °C; (3) 60-s extension at 72 °C and a final extension for 8 min at 72 °C. The amplified PCR products were purified using Qiagen PCR purification kit. To validate the single-nucleotide polymorphisms for cytotyping, we sequenced purified products on ABI 3730XL DNA analyzer (Life Technologies, Carlsbad, CA, USA). For dCAPs, we digested 6 μl of purified *ndh*F product from each sample with five units of the restriction enzyme *Msl*I for 2 h at 37 °C, and 6 μl of purified *pet*D product with five units of *Taq*^{α}I at 65 °C for 2 h. The recognition site of *Msl*I includes the underlined variable site (CATTG^AAGTA/CATTGAAGT<u>G</u>) within *ndh*F and that of *Taq*^{α}I includes the underlined variable site (T^C<u>G</u>A/TC<u>A</u>A) in *pet*D. Products were assayed on agarose gels and species-specific cytotypes identified as follows: (1) *ndh*F locus, a 500-bp (uncut) product identifies *F. virginiana* whereas two fragments (336 and 166-bp product) corresponds to the *F. chiloensis* cytotype; (2) two *pet*D fragments (56 and 44 bp) differentiate the *F. chiloensis* cytotype from the *F. virginiana* (uncut) cytotype.

Nuclear marker analysis and genotyping

As we were interested in determining the location of sex-determining region and assessing the homology of the sex-determining chromosome in *F. × ananassa* subsp. *cuneifolia* to its progenitors, we genotyped the mapping populations using primer pairs that have been shown to amplify DNA markers (simple sequence repeats or genes) on LG within the homoeologous group (HG) that houses the sex-determining chromosomes in *F. virginiana* and *F. chiloensis* (that is, HG VI, Goldberg *et al.*, 2010; Spigler *et al.*, 2010). Thirty-nine primer pairs were used to construct genetic map of HG VI and map the sex-determining region in *F. × ananassa* subsp. *cuneifolia* in the WREN7 × MP10 cross and 10 of these were used to construct genetic map for the MP12 × WREN2 cross.

For nuclear markers, PCR reactions were performed using the Poor Man's PCR protocol as previously described (Spigler *et al.*, 2008; Goldberg *et al.*, 2010). We multiplexed PCR products from 2 to 4 primers by mixing 1.3 μl aliquots from each reaction with 0.2 μl LIZ500 standard and 10.5 μl Hi-Di formamide (Applied Biosystems). Fragment analysis and genotyping were conducted using ABI 3730XL DNA analyzer and GeneMapper ver 3.0 (Applied Biosystems, Life Technologies, Carlsbad, CA, USA).

Construction of genetic maps

We used the single-dose restriction fragment marker analysis and a pseudo test-cross strategy to construct genetic linkage maps for *F. × ananassa* subsp. *cuneifolia*, as is customary in polyploids (Wu *et al.*, 1992; Garcia *et al.*, 2006). As a single-primer pair can amplify multiple PCR products as a result of paralogs in the octoploid genome, we considered each PCR product a single-dose marker and scored it as present (1) and absent (0) in the progeny. Using χ^2 tests, we evaluated each polymorphic marker for goodness of fit according to the expected Mendelian segregation ratio of either 1:1 if present in only one parent or 3:1 if present in both parents. In each cross, we discarded markers that deviated from the expected segregation ratios at $P \geqslant 0.0001$ and retained all other markers for mapping. We mapped the marker data from each primer pair in JoinMap 4.0 (Van Ooijen, 2006) to determine whether markers from a given primer pair represented co-segregating alleles at a single locus. The PCR products from single primer were considered to be allelic at a locus if they mapped in the same location and were in repulsion. For these, we retained one member of each pair to be consistent with the single-dose marker approach

(Wu *et al.*, 1992; Spigler *et al.*, 2010). For PCR products that were linked but did not map to the same location, we checked the raw data to reevaluate the genotypes and reconfirm scores as in past work (Spigler *et al.*, 2010).

We constructed separate maps of HG VI for maternal and paternal parents by considering them as cross-pollinator population type (outbred full-sib family cross). Before mapping, we excluded markers or individuals that were missing data for >25%. This resulted in the exclusion of only two individuals from one cross (MP12 × WREN2). Initial LGs were inferred at logarithm of odds (LOD) $\geqslant 6$. Additional ungrouped markers were assigned at LOD >4 threshold using strongest cross-link values. Marker order and map distance were determined using the Kosambi mapping function with default parameter settings (minimum LOD threshold of 1.0, recombination threshold of 0.40 and jump threshold of 5.0) to include maximum number of markers to identify homoeologs within HG VI. We also applied strict mapping parameters (minimum LOD threshold of 3.0, recombination threshold of 0.35 and jump threshold of 3.0) to test stability of linkage between markers within each homoeolog and found no major differences from the less strict mapping parameter settings. We considered genetic maps derived from default mapping parameter for further analyses. Graphical maps were constructed in MapChart 2.1 (Voorrips, 2002).

The LGs within each parental HG VI map were assembled on the basis of strongest cross-link values of LOD $\geqslant 4$ between markers on the LGs or by comparing the LG with a putative homoeolog in the other parent (based on shared markers fitting 3:1 segregation ratio). LGs were named VI.A, VI.B, VI.C and VI.D based on the LG-specific markers on homoeologous LGs in *F. virginiana* and *F. chiloensis* genetic maps (Goldberg *et al.*, 2010; Spigler *et al.*, 2010). Mapped markers that deviated in expected segregation ratios at $0.0001 < P < 0.01$ were identified as skewed and are denoted along with the direction of skew (that is, under or over representation of the heterozygote).

Qualitative and quantitative mapping of sex expression

To qualitatively map sex function traits, we considered male sterility and female sterility as single-dose markers and tested for fit to Mendelian segregation ratios (1:1 and 3:1) as in past work (Spigler *et al.*, 2008; Goldberg *et al.*, 2010).

To quantitatively map female function, the mapping populations were treated as 'doubled haploid populations', which can evaluate quantitative trait locus (QTL) more efficiently (Van Ooijen, 2004), but require the exclusion of markers found in both parents that segregate 3:1 (that is, 'hk × hk' segregation types in JoinMap) from the genetic map (Van Ooijen, 2006). We conducted QTL analysis on each parent map separately using MapQTL5 (Van Ooijen, 2004). As in past work (Spigler *et al.*, 2010, Ashman *et al.*, 2011), we used Kruskal−Wallace analysis followed by interval mapping and composite interval mapping (multiple QTL model, 'MQM', in MapQTL) to identify potential single-marker associations and QTL for fruit set. Only the markers identified as significant in the MQM were also significant in the single-marker tests after Bonferroni correction, thus we only report the results of the more conservative MQM results. The QTLs (delineated by 2-LOD intervals) detected through MQM are depicted on the original LG map for simplicity using an autoqtl function in MapChart (Voorrips, 2002). Results were unchanged when QTL analysis was performed without the 'marker' male sterility (data not shown). We tested for epistatic effects between male sterility and female function QTL when they did not overlap, as well as between multiple female function QTL when present, following the analysis of variance approach of Spigler *et al.* (2011).

To assess macrosynteny of the sex-determining chromosomes in *F. × ananassa* subsp. *cuneifolia* with the parental species, we aligned the LGs carrying male sterility found here with those published for *F. virginiana* and *F. chiloensis* (Goldberg *et al.*, 2010; Spigler *et al.*, 2010). We also included the homoeolog from a cross between two diploid hermaphrodite species, *Fragaria vesca* and *Fragaria nubicola* ('Fv × Fn', Sargent *et al.*, 2009) for reference.

RESULTS

Cytotyping

Both *F. × ananassa* subsp. *cuneifolia* populations contained *F. chiloensis* and *F. virginiana* cytotypes, but they differed dramatically

Table 1 Frequency of *F. chiloensis* and *F. virginiana* cytotypes found in two *F.* × *ananassa* subsp. *cuneifolia* populations

Population	Cytotype	Gene		Consensus	
		petD	*ndhF*	*Total*	*%*
MP	*F. chiloensis*	29	29	29	93.5
	F. virginiana	2	2	2	6.5
WREN	*F. chiloensis*	2	2	2	6.6
	F. virginiana	30	27	30	93.4

Abbreviation: MP, Mary's Peak.

in the frequency of the two types (Table 1). The *F. chiloensis* cytotype was predominant in the MP population (93.5%) whereas the *F. virginiana* one was at Wren (93.4%). The parents of the mapping populations had the majority cytotypes of their respective populations.

Genetic maps of HG-VI

MP12 × WREN2. Ten primer pairs amplified 75 PCR products, 57 of which met our criteria for map construction. Five of the 25 markers (19 (1:1 markers) and 6 (3:1 markers)) used for the maternal map co-segregated so 20 markers were retained for mapping. In the paternal map, 12 of the 36 markers (30 (1:1 markers) and 6 (3:1 markers)) co-segregated, thus 24 were retained for mapping. From these, seven and three markers (maternal and paternal, respectively) were unlinked and not included in the final map.

We assembled the LGs of the maternal map (Supplementary Figure 1, top) into two homoeologous chromosomes (VI.A and VI.B) based on synteny with the progenitor species and the WREN map (see below). The size of LGs ranged from 20 to 66.2 cM, with 4 to 7 markers per group.

The paternal map (Supplementary Figure 1, bottom) was resolved into five LGs that were assembled into three homoeologous chromosomes (VI.A, VI.B and VI.C). The size of the LGs ranged from 1.2 to 41.1 cM, with 2 to 8 markers per group. In the paternal map only, 14% of the 21 mapped markers were skewed. There were three single markers (SCAR2, CFCVT017 and UFFxa01E03) that showed moderate segregation distortion ($P < 0.01$) and over-representation of heterozygous gametes among genotypes.

WREN7 × MP10. Thirty-nine primer pairs amplified 225 products. From these, 142 met our criteria for genetic map construction. In total, 13 of the 79 markers (54 (1:1 markers) and 25 (3:1 markers)) used for maternal map co-segregated, so 66 were retained for final mapping. In the paternal map, 16 of the 88 markers (63 (1:1 markers) and 25 (3:1 markers)) co-segregated and thus 72 were retained for mapping. In all, 25 and 30 markers of the final sets were unlinked (maternal and paternal, respectively), thus are not included in the final maps.

The maternal map of HG VI (Supplementary Figure 2, top) comprises 41 markers that resolved into 8 LGs. We assembled six of these LGs into four homoeologous chromosomes and identified LGs VI.A through VI.D based on the presence of LG-specific markers in comparison with the progenitor genetic maps. For instance, SCAR2 on VI.A, F.v.a108 on VI.C, F.v.B119 on VI.D in both *F. virginiana* and *F. chiloensis* maps (Goldberg *et al.*, 2010; Spigler *et al.*, 2010) and tandemly duplicated EMFv104 markers along with other markers as LG VI.B in *F. chiloensis* (Goldberg *et al.*, 2010). These LGs ranged in size from 13.4 to 82.1 cM, with 3 to 17 markers per group. Two small

LGs (two markers) were not assigned to a homoeologous chromosome. Only one confirmed case of a duplicated marker was found (CFVCT006 on LG VI.A).

The paternal map of HG VI (Supplementary Figure 2, bottom) included 42 markers and resolved into 7 LGs, which we assembled into 4 LGs homologous to the maternal map. Three small LGs (two markers each) were not assigned to homoeologous chromosomes. LGs had 5 to 17 markers per group and ranged from 25.7 to 90 cM.

In this cross, 16 (19%) of the mapped markers did not fit the expected Mendelian ratios. Of these, 69% where paternal markers whereas 31% where maternal ones. These were most often single markers (Supplementary Figure 2, bottom). One potentially interesting case, however, is the two products of CFCVT017 on LG VI.B close to the QTL for fruit set in both maternal and paternal maps that showed a modest ($P < 0.01$) under-representation of heterozygous gametes among genotypes.

Phenotypic sex expression: variation and mapping

Phenotypic sex expression. Of the 85 genotyped progeny from MP12 × WREN2 cross, 40 progeny were male sterile and 45 were male fertile, thus male function segregated 1:1 (Figure 2a; $\chi^2 = 0.30$, $P = 0.59$). In this cross, female function also segregated 1:1 ($\chi^2 = 0.42$, $P = 0.51$) as 46 were female fertile and 39 were female sterile. The majority (92%) of progeny were either female (male sterile and female fertile; 47%), or male (male fertile and female sterile; 45%), and six were hermaphrodites. Six females had fruit set lower than 75% (Figure 2a).

In the WREN7 × MP10 cross, 47 of the genotyped progeny were male sterile and 43 were male fertile, thus male function segregated 1:1 (Figure 2b; $\chi^2 = 0.17$, $P = 0.67$) in this cross as well. When female function was scored qualitatively, 92% of progeny were female fertile and female fertility deviated significantly from both 1:1 and 3:1 (both $P < 0.0001$), precluding qualitative mapping (see below). Overall, 36 (40%) progeny were hermaphrodite, 47 (52%) female and 7 (0.07%) male (Figure 2b).

Male function mapping. The results from both the crosses confirm the dominance of male sterility over male fertility, and in both map crosses male sterility mapped to one of the homoeologs within HG VI (Figure 1; Supplementary Figures 1 and 2). In MP12 × WREN2, male sterility mapped to the bottom of VI.A with tight linkage to two markers, PSContig6115 (LOD 10.0) and EMFn153 (LOD 5.1). In WREN7 × MP10, male sterility was linked in coupling to EMFv104_143 at a very high LOD (21.4). This marker is one of three tandemly duplicated products of the EMFv104 primer pair on LG VI.B (for example, EMFv104_143, EMFv104_135 and EMFv104_133).

Female function mapping. Qualitative mapping of female function in MP12 × WREN2 indicated that female function was linked to male sterility (Figure 1a). Similar to that seen in *F. chiloensis* (Goldberg *et al.*, 2010), female fertility was dominant to female sterility and mapped in coupling with male sterility in the maternal parent. The QTL analysis of proportion fruit set is consistent with the qualitative mapping in this cross. A major QTL for proportion fruit set was found on LG VI.A in maternal map with a LOD score of 46.8 in MQM (Supplementary Figure 1). This QTL explained 93.2% of the variation in fruit set and its peak was within 1.27 cM of male sterility.

In the WREN7 × MP10 cross, only a quantitative approach to mapping female function was possible. In the maternal map, a QTL that explained 91.3% variation in fruit-setting ability colocalized with male sterility on LG VI.B with a LOD score of 47.2 (Supplementary
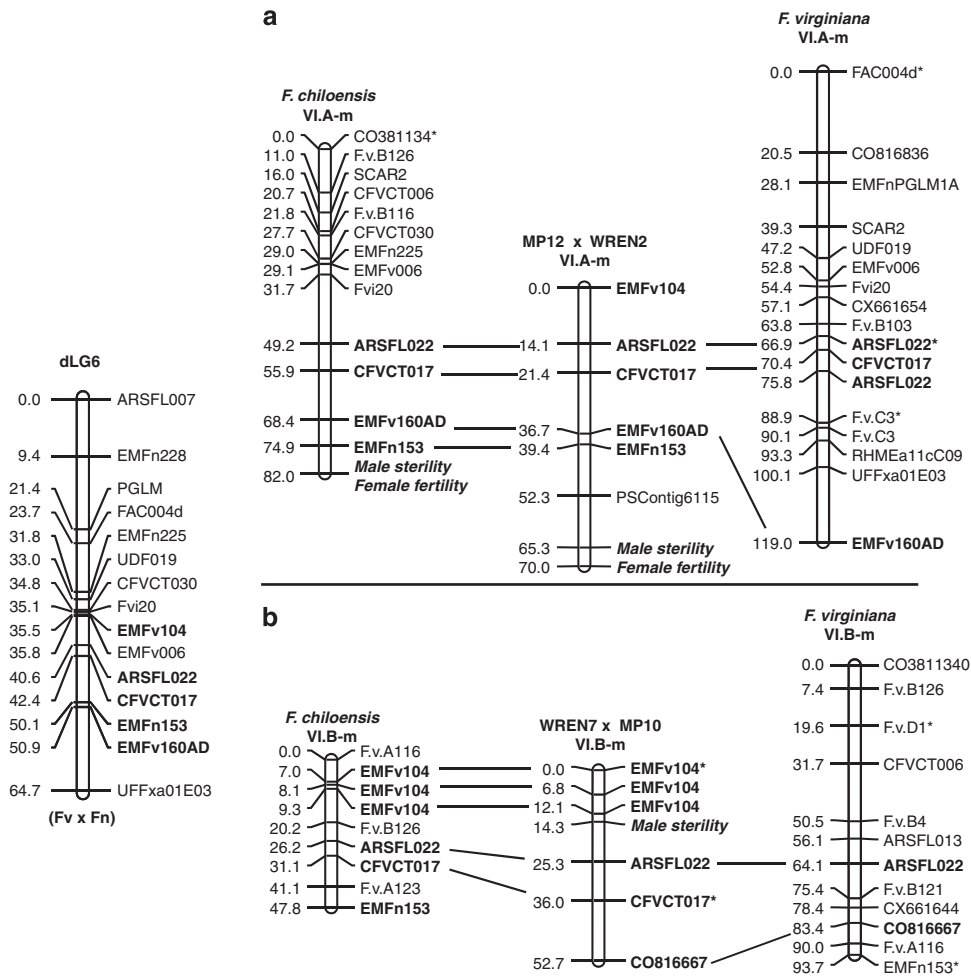
Figure 1 Comparison of male sterility carrying sex-determining chromosomes from both cross (**a**, **b**) to the corresponding homoeologs of *F. virginiana* (Spigler *et al.*, 2010) and *F. chiloensis* (Goldberg *et al.*, 2010) and the homoeologous LG6 in hermaphroditic diploid cross (Fv × Fn, adapted from Sargent *et al.*, 2009) from previously published maps. Simple sequence repeats on the corresponding homoeologs in octoploids are connected by lines and are highlighted in bold to indicate synteny with the diploid reference homoeologous LG (dLG6). Phenotypic trait marker representing the putative sex determining loci (male sterility/female fertility) are indicated in bold and italicized. Markers denoted by asterisk had skewed segregation ratios (0.0001 < *P* < 0.01).

Figure 2). In the paternal map, a QTL explaining 10.6% variation for fruit set was linked to CFVCT017 with LOD score of 1.89 and this QTL had a negative additive effect on fruit set. However, no significant epistatic interaction was found between this QTL and the female function QTL in the maternal parent when tested using analysis of variance (*P* > 0.05).

Macrosynteny of sex-determining chromosomes
We compared the linkage map of the male sterility-determining chromosomes with their homoeologs in *F. virginiana* and *F. chiloensis* and in hermaphrodite diploid cross (dLG 6) (Figure 1). This revealed macrosynteny and only small differences in colinearity among these LGs. For instance, although the linkage map of the sex-determining chromosomes from both *F.* × *ananassa* subsp. *cuneifolia* crosses share three to five markers with LG 6 in diploid Fv × Fn cross, the order of EMFv160AD and EMFn153 in the MP12 × WREN2 cross agrees with the order in *F. chiloensis* but not Fv × Fn (Figure 1a). The synteny with *F. chiloensis* is in accord with fact that the maternal parent (MP12) has the *F. chiloensis* cytotype. A notable difference, however, is the greater estimated distance (30 vs 7 cM) between the marker EMFn153 and male sterility in the *F.* × *ananassa* subsp. *cuneifolia*

cross than in the *F. chiloensis* cross (Goldberg *et al.*, 2010). In WREN7 × MP10, male sterility mapped above the three markers (ARSFL022, CFVCT017 and EMFn153) that subtended male sterility in MP12 × WREN2 and very close to a set of tandemly duplicated EMFv104 markers on LG VI.B. This location is not in agreement with the expected location of male sterility (on the tip of LG VI.C) given the *F. virginiana* cytotype of the maternal parent (WREN7). The tandemly duplicated set of EMFv104 markers, however, is similar to their arrangement on LG VI.B in *F. chiloensis* (Figure 1b; Goldberg *et al.*, 2010) an alignment not observed in *F. virginiana* (Figure 1b; Spigler *et al.*, 2010). Two markers located below male sterility (ARSFL022, CFVCT017) are shared by LG VI.B in *F. chiloensis* and two (ARSFL022, CO816667) by LG VI.B in *F. virginiana* (Figure 1b).

Sexual dimorphism
Proportion fruit set and proportion seed set were strongly sexually dimorphic in the progeny of both *F.* × *ananassa* subsp. *cuneifolia* crosses while ovule number was dimorphic in one cross (Table 2). In contrast, sexual dimorphism was nonexistent in anther number, runner number and leaf number, and was only weakly (*P* < 0.08) dimorphic for flower number in the WREN7 × MP10 cross (Table 2).

The direction of dimorphism for flower number (male-sterile > male-fertile morphs) was opposite of that published for either of the pure species crosses (male-sterile < male-fertile morphs; Ashman *et al.*, 2011; Spigler *et al.*, 2011), as was the direction of dimorphism for ovule number in the WREN7 × MP10 cross (male-sterile < male-fertile morphs; Ashman and Hitchens, 2000; Spigler *et al.*, 2011).

DISCUSSION

Cytotypic composition of *F.* × *ananassa* subsp. *cuneifolia* hybrid populations

Our results show for the first time that natural hybrid populations of *F.* × *ananassa* subsp. *cuneifolia* contain cytotypes from both maternal

Figure 2 Frequency histograms of proportion fruit set (female function) for male sterile (MS) and male fertile (MF) progeny of *F.* × *ananassa* subsp. *cuneifolia* crosses. (**a**) MP12 × WREN2. (**b**) WREN7 × MP10 cross.

species but that individual populations show a strong bias toward one cytotype or the other. The presence of both cytotypes suggests that both species contributed as maternal parents to the studied populations, and that admixture is still occurring, or that there was paternal leakage, although the latter is very rare in flowering plants (for example, McCauley *et al.*, 2007) and was not detected in our crosses (unpublished data). Extreme bias in cytotypes is not uncommon in hybrid species, and can reflect the signature of the historical range of the dominant maternal species or of selective forces that favor one cytotype (Minder *et al.*, 2007; Arnold *et al.*, 2010). The two populations here are 13 km apart and are both near the margin of *F. virginiana*'s range, although the MP population is closer to the coast. On the other hand, the MP population is on the highest peak (1249 m) in the Oregon Coast Range, an unusual habitat for *F. chiloensis* and thus could reflect a remnant population, or a long distance migrant, which was then subject to gene flow from *F. virginiana*. Broad sampling is underway to determine whether there is a geographic or edaphic pattern to the dominance of maternal cytotype and to assess the extent and direction of admixture in the nuclear genome across the hybrid zone. Such work will provide a landscape assessment of introgressive hybridization between *F. chiloensis* and *F. virginiana*, as well as indicate the potential for spread of new sterility alleles across the hybrid zone to contribute to turnover in sex-determining chromosomes in the two parental octoploid species (Veltsos *et al.*, 2008). Reciprocal transplant and selection analyses will also provide powerful means to assess fertility selection on male and female function (for example, Spigler and Ashman, 2011) in the context of the sex expression variation provided by the hybrid zone (see below).
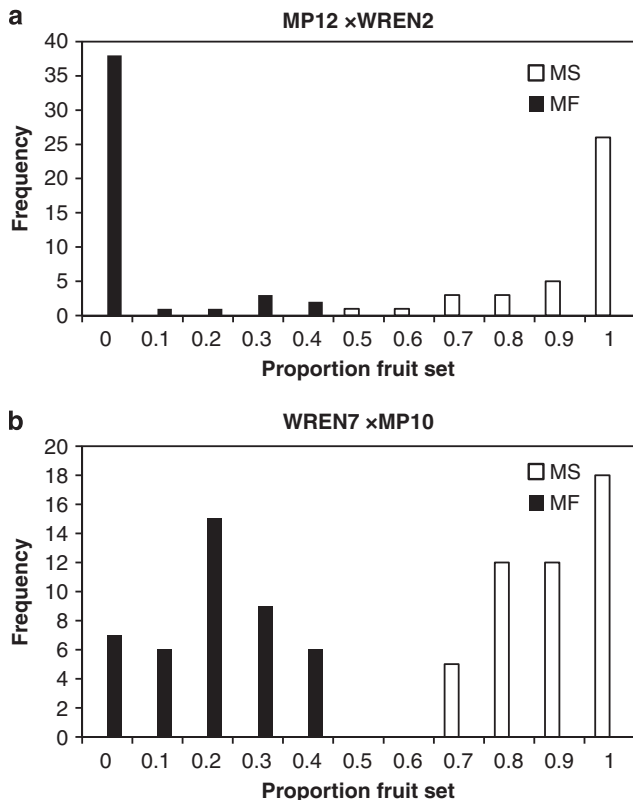
Variation in sex determination regions of *F.* × *ananassa* subsp. *cuneifolia* crosses

Although there was variation in the location of sex function loci, major QTL were found in HG VI, confirming the important role of this HG in sex determination in the octoploid *Fragaria* species. A similar consistent involvement of a specific LG has been seen among *Populus* species, that is, all sex-containing *Populus* linkage maps show sex determination on LG XIX, although in different positions and sometimes segregating in different parental genders (Pakull *et al.*, 2009).

F. × *ananassa* subsp. *cuneifolia* is similar to both its parental species in that male sterility mapped in the female parent and was dominant to male fertility in both crosses. In one cross (MP12 × WREN2), male sterility mapped to the location predicted by maternal cytotype

Table 2 Trait means (± s.e.) for MS and MF F1 progeny in the *F.* × *ananassa* subsp. *cuneifolia* map crosses and SD indices

Trait	MP12 × WREN2				WREN7 × MP10			
	MS	MF	P[a]	SD index[b]	MS	MF	P[a]	SD index[b]
Proportion fruit set	0.89 (0.20)	0.04 (0.11)	<0.0001	35.81	0.84 (0.11)	0.16 (0.11)	<0.0001	43.25
Proportion seed set	0.74 (0.25)	0.13 (0.30)	<0.0001	14.59	0.88 (0.10)	0.64 (0.34)	<0.0001	7.43
Flower number	7.0 (3.6)	8.2 (3.8)	0.16	2.02	16.0 (5.3)	14.0 (5.2)	0.08	2.55
Ovule number	52.0 (11.9)	50.4 (15.0)	0.58	0.79	55.0 (11.6)	64.7 (14.2)	<0.005	5.06
Anther number	20.7 (4.1)	20.2 (3.7)	0.53	0.89	20.2 (1.8)	20.2 (1.3)	0.96	0.08
Leaf number	10.8 (4.4)	11.7 (4.6)	0.35	1.33	18.0 (7.1)	18.5 (7.7)	0.75	0.46
Runner number	6.3 (2.1)	6.4 (2.2)	0.72	0.51	5.0 (2.2)	5.1 (2.3)	0.88	0.21

Abbreviations: MF, male-fertile; MP, Mary's Peak; MS, male-sterile; SD, sexual dimorphism.
[a]Progeny means were evaluated using *t*-test and *P* < 0.05 indicated in bold.
[b]SD index = |($x_{MS} - x_{MF}$)|/[(s.e.$_{MS}$ + s.e.$_{MF}$)/2], where *x* and s.e. are the mean and standard error, respectively, for each trait MS and MF progeny.

(*F. chiloensis*), but in the other cross (WREN7 × MP10) male sterility mapped to a novel location. The novel location could be the result of transposition during hybrid formation as such rearrangements can be common in some hybrids (for example, Lai *et al.*, 2005). Alternately, it is possible that male sterility has a different location in the western subspecies of *F. virginiana* subsp. *platypetala* than in the eastern *F. virginiana* subsp. *virginiana*. Current hypotheses for the biogeographic history of *F. virginiana* suggest that it, like many species in North America, may have been separated into two vicarious groups as the result of uplifting of the Rocky Mountains (Staudt, 1999). Using morphological and RAPD data, Harrison *et al.* (1997) concluded that *F. virginiana* subsp. *platypetala* was substantially differentiated from the rest of *F. virginiana,* and was more closely related to *F. chiloensis* than to *F. virginiana* subsp. *virginiana*. Staudt (1999) also suggested that *F. virginiana* subsp. *platypetala* may itself been derived from recent hybrids of *F. virginiana* and *F. chiloensis*. Although there is little consensus regarding these designations (Hancock *et al.*, 2004), data to date do fuel speculation that gene flow between *F. virginiana* and *F. chiloensis* could be responsible for the dynamic nature of the sex-determining region. Future work mapping sex determination within *F. virginiana* subsp. *platypetala* and work underway characterizing the extent of admixture in the nuclear genome across the hybrid zone will help resolve these issues.

Female function was found to map to a single region (and female sterility was recessive to female fertility) in one cross but mapped to two regions in the other cross. A single major QTL for female function in MP12 × WREN2 was linked to male function on VI.A, consistent with the location and linkage phase in the genetic map of *F. chiloensis* (male sterility in coupling with female fertility; Goldberg *et al.*, 2010), but the linkage between the two was not as tight and recombinants were formed. In this cross, we can infer the genotypes of the parents as *AG|ag* for the maternal parent and *ag|ag* for the paternal parent, creating a majority of non-recombinant male and female progeny, and a smaller fraction of recombinant progeny, that is, the low fruit-setting females and hermaphrodites (Figure 2a; Supplementary Table 1a). This also conforms to Charlesworth and Charlesworth's (1978) two-locus model for sex determination (also see Spigler *et al.* 2008).

Not only was the location of male sterility in WREN7 × MP10 not as predicted, but the finding of two QTL for female sterility was unexpected. Multiple QTL affecting female function could indicate that (1) rearrangements of loci in the hybrid has led to novel placements of existing loci or (2) the hybrid has unique female-sterility alleles. We discuss the present results in the context of these two possibilities.

The QTL for female function linked to EMFv104 on VI.B clearly represents a new location relative to published maps of parental species and coincides with the QTL for male sterility, a location that could reflect either a transposition or new sterility genes as the result of hybridization, or as mentioned above a difference in the location of the sex-determining genes derived from the western subspecies of *F. virginiana*. If transposition occurred, it may have involved linked genes or a single gene that has pleiotropic effect on both male and female function. Determining this would require finer mapping and a larger population size, as there is no clear evidence in our current map for any other transposed markers in this region.

The QTL for fruit set overlapping CVCT017 (and EMFn153) on LG VI.B in the paternal parent, could also represent a transposition of a QTL, possibly from VI.A, as the paternal parent has *F. chiloensis* cytotype, or given the allopolyploid origin (for example, AA A′A′BB B′B′) of the parental species could represent an orthologous QTL

affecting fruit set on VI.B, that is, from a different genome donor, or a novel sterility locus. An orthologous QTL is a viable hypothesis because a recent study of the cultivated hybrid strawberry (*F.* × *ananassa* subsp. *ananassa*) concluded that ~25% of QTL for fruit traits were at orthologous positions on a different homoeologous LGs, that is, were putative 'homoeo-QTL' (Lerceteau-Köhler *et al.*, 2012), and these may be segregating in the natural hybrid species as well. Moreover, there is no evidence for transposition of other markers, which might be expected if nonhomologous recombination triggered the QTL at this position. In fact, there was only one confirmed case of novel duplication (CFVCT006 on LG VI.A) in our map of the natural hybrid.

However, sterility can directly result from hybridization (reviewed in Maheshwari and Barbash, 2011). Of the many possible mechanisms, two have some support in plants: (1) negative genetic interactions between two or more loci fixed in the two parental species (for example, Dobzhansky−Muller incompatibilities; Fishman and Willis, 2001; Moyle and Nakazato, 2010; Maheshwari and Barbash, 2011), and (2) rearrangements (for example, pollen sterility QTL were located near rearrangement breakpoints in artificial *Helianthus* hybrids; Lai *et al.*, 2005). Both are possibilities here because the characteristics of sex chromosomes, in particular, are thought to make them 'hotspots' for speciation genes (Qvarnstrom and Bailey, 2008), and rearrangements have been found in the octoploid species that involve LGs in HG VI (Spigler *et al.*, 2010; Sargent *et al.*, 2012). The negative additive effect of the fruit set QTL in paternal parent (reduces fruit set by 10%) and the skewed segregation of the nearest marker (Supplementary Figure 1) might indicate the involvement of a Dobzhansky−Muller incompatibility. Further crosses are required to resolve whether female sterility at this location is the manifestation of different genes related to speciation in this region.

In the context of past models (Spigler *et al.*, 2008; Goldberg *et al.*, 2010), the diversity of sexual phenotypes in the WREN (Figure 2), including low fruiting females and moderately fruiting hermaphrodites, can be accounted for by recombination between the two female function QTL on LG VI.B (Supplementary Table 1B).

Sexual dimorphism in hybrid *F.* × *ananassa* subsp. *cuneifolia*

Aside from components of female function (fruit set and seed set), other traits that have been found to be significantly sexually dimorphic in the pure species were either not dimorphic in the hybrid crosses (anther number, runner number and leaf number, flower number; Staudt, 1999; Ashman *et al.*, 2011; Spigler *et al.*, 2011) or if they were dimorphic the direction of dimorphism was in the opposite to what has been observed in the pure species (ovule number and flower number in the WREN7 × MP10 cross; Table 2). These results suggest that hybrids experience some disruption of dimorphism in secondary sexual traits. Loss of dimorphism in hybrids has been interpreted as reflecting breakdown of modifiers or regulatory elements that are responsible for dimorphism and suggests that these traits were originally expressed in both sexes but that modifier evolved afterward (Coyne *et al.*, 2008). This interpretation may also apply to the traits studied here because all are expressed in both sexes to some degree and modifiers may be linked to sex-determining region (Spigler *et al.*, 2011). In one of the few studies to assess dimorphism after hybridization, Zluvova *et al.* (2005) found females from crosses between dioecious *Silene latifolia* and hermaphrodite *Silene viscosa* had anthers that developed beyond the stage characteristic for *S. latifolia* females. Thus, the hybrid had less dimorphism in anther size than the pure species, and they interpreted this as evidence that the suppression of anthers was brought about by a recessive allele that

could be rescued by the genome of *S. viscosa*. However, they did not find the same effect for fruit set in the males. Our work is far from conclusive, but does suggest that studies of the effects of hybridization on sexual dimorphism in plants will be useful for gaining insight into the genetic underpinnings and evolutionary processes (Coyne *et al.*, 2008) and will be exemplary complements to studies of early sex chromosome evolution in plants.

CONCLUSION

The work presented here suggests that hybrid zones of dioecious/subdioecious plants are valuable and underutilized resource for studying the ecology and evolution of sex chromosomes and sexual dimorphism. Ecological work will be especially valuable to test hypotheses for the spread of new chromosomal sex determination systems across hybrid populations (Veltsos *et al.*, 2008). In addition, surveys of sexual dimorphism in natural hybrid zones, along with the creation of experimental hybrids will provide novel insight into the evolution and control of sexual dimorphism during the evolution of sex chromosomes.

DATA ARCHIVING

Sequence data have been submitted to GenBank: JX064433-JX064440; JX064449-JX064456. Genotype and phenotype data are deposited with Dryad: doi:10.5061/dryad.cn66t. Data files: Cuneifolia_data_files_readme_files.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)