Sex Determination in the Androdioecious Plant *Datisca glomerata* and Its Dioecious Sister Species *D. cannabina*

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ABSTRACT

Datisca glomerata is an androdioecious plant species containing male and hermaphroditic individuals. Molecular markers and crossing data suggest that, in both *D. glomerata* and its dioecious sister species *D. cannabina*, sex is determined by a single nuclear locus, at which maleness is dominant. Supporting this conclusion, an amplified fragment length polymorphism (AFLP) is heterozygous in males and homozygous recessive in hermaphrodites in three populations of the androdioecious species. Additionally, hermaphrodite × male crosses produced 1:1 sex ratios, while hermaphrodite × hermaphrodite crosses produced almost entirely hermaphroditic offspring. No perfectly sex-linked marker was found in the dioecious species, but all markers associated with sex mapped to a single linkage group and were heterozygous in the male parent. There was no sex-ratio heterogeneity among crosses within *D. cannabina* collections, but males from one collection produced highly biased sex ratios (94% females), suggesting that there may be sex-linked meiotic drive or a cytoplasmic sex-ratio factor. Interspecific crosses produced only male and female offspring, but no hermaphrodites, suggesting that hermaphroditism is recessive to femaleness. This comparative approach suggests that the hermaphrodite form arose in a dioecious population from a recessive mutation that allowed females to produce pollen.

ANDRODIOECY is a rare and unusual breeding system in which populations contain both male and hermaphroditic individuals. In this article, we examined the genetic basis of sex determination in an androdioecious plant species *Datisca glomerata* and its dioecious sister species *D. cannabina*.

Although androdioecy is rare, its maintenance and evolution have broad implications for breeding system theory in general (Charlesworth 1993). Because hermaphroditism is thought to be the ancestral breeding system of angiosperms (Darwin 1877; Lewis 1942; Bawa 1980; Charlesworth 1985), previous attempts to model the evolution of androdioecy have assumed that it arose from hermaphroditism after the invasion of a female-sterile mutant conferring maleness (Ross and Weir 1976; Charlesworth and Charlesworth 1978; Charlesworth 1984). For a male to invade a hermaphroditic population, the new mutant must achieve at least twice as many fertilizations as hermaphrodites and even more if hermaphrodites partially self-fertilize (Lloyd 1975; Charlesworth and Charlesworth 1978; Charlesworth 1984). These authors agreed that a new mutant eliminating female function was unlikely to simultaneously double its fitness via male function, and therefore androdioecy was unlikely to evolve by this mechanism.

More recent evidence, however, suggests that many cases of androdioecy (and near-androdioecy) have evolved from dioecy (males and females) rather than from hermaphroditism. Phylogenetic evidence suggests that *Eulimnadia texana* (Sassaman 1995), *Caenorhabditis elegans* (Fitch and Emmons 1995; Fitch *et al.* 1995), and *Datisca glomerata* (Rieseberg *et al.* 1992; Swensen *et al.* 1998) are all derived from dioecious systems. Observations that the majority of congeners are dioecious suggest a dioecious origin in *Mercurialis annua* (Pannell 1997c) and *Schizopepon bryoniaefolius* (Akimoto *et al.* 1999). Therefore, comparisons between androdioecious species and their dioecious relatives can provide insight into the evolution of androdioecy.

Additionally, an understanding of sex determination in androdioecious species may help us to understand the evolution and maintenance of androdioecy. In particular, dominance relationships are likely to influence the evolution and maintenance of androdioecy. In the absence of inbreeding, beneficial mutations are more likely to invade a population if they are dominant than if they are recessive (Haldane 1927; Bodmer and Parsons 1960). Thus, a dominant mutation for hermaphroditism may be more likely than a recessive mutation to invade a dioecious population and lead to androdioecy. Dominance relationships can also influence the probability with which androdioecy is maintained, when meta-

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population dynamics are considered: Androdioecy is more likely to be maintained under high rates of extinction and recolonization if maleness is dominant to hermaphroditism, whereas androdioecy is more stable under low rates of extinction and recolonization if maleness is recessive (Pannell 1997c). Likewise, the number of independently segregating sex-determining loci may influence the frequency at which male-determining alleles can be maintained and the conditions under which maleness is expressed, therefore influencing the maintenance of androdioecy.

Another reason to study sex determination is to allow confirmation that morphs in a seemingly androdioecious system are genetically determined, rather than conditionally expressed phases of cosexuality. The conditions required for the evolution and maintenance of androdioecy are likely to be rare in nature (Lloyd 1975; Charlesworth 1984), but the conditions favoring gender switching in cosexual populations are common (Charnov and Bull 1989; Charnov and Dawson 1989; Clay 1993). Thus, it is necessary to demonstrate the presence of a genetically distinct male morph before confirming that a species is truly androdioecious. Data reported in this article suggest that males and hermaphrodites are genetically distinct in *D. glomerata*.

One approach to understanding the number of loci controlling a trait and dominance relationships at those loci is through controlled crosses. However interpretations from crosses alone are often inconclusive and can be confounded by complex forms of inheritance and non-Mendelian factors, such as segregation distortion and environmental effects. Sex-linked segregation distortion is common, and biased sex ratios are observed even in organisms with sex chromosomes. Mechanisms that cause biased sex ratios in controlled crosses include differential success of X- and Y-bearing gametes during pollen competition (Correns 1928; Lloyd 1974; Lassere *et al.* 1996), maternal factors influencing pollen growth (Miglia and Freeman 1996), differential seed germination (Lyons *et al.* 1995; Purrington and Schmitt 1995), differential survival and flowering (Allen and Antos 1993), and X-linked or cytoplasmic factors (Taylor 1994a). Cytoplasmic factors are well studied in invertebrates and frequently consist of maternally inherited parasites that kill or feminize males (Hurst 1993; Juchault *et al.* 1994). Likewise, sex-linked meiotic drive, along with genes that repress meiotic drive are common and well studied in both plants and animals (Lyttle 1991; Carvalho and Klaczko 1994; Taylor 1999).

These examples suggest that the factors controlling sex ratios may or may not be related to factors that determine the sex of individuals. A simple nuclear system may determine the sex of individuals, even if some other factor determines sex ratios. In such cases, cytological data or molecular markers are necessary to expose the underlying sex-determining factors.

Deciphering the genetic basis of sex determination in species with heteromorphic sex chromosomes is generally straightforward. Similarly, when heteromorphic chromosomes cannot be identified, as is the case in Datisca (J. Qiu, L. H. Rieseberg and T. Philbrick, unpublished results), molecular markers can be used to track the inheritance of chromosomal segments and nearby sex-determining loci. Investigation of sex and marker segregation in controlled crosses can then reveal the number of loci involved in sex determination and the dominance relationships among alleles. When there is a single segregating sex-determining locus, closely linked markers will cosegregate with the sexual phenotype. Markers are not expected to tightly cosegregate with sex if there are multiple, unlinked sex-determining loci. Nonetheless, weak associations between sex and markers near the sex-determining loci are expected, in which case a linkage-mapping approach can be used to make inferences about the genetic architecture of sex determination.

This article examines the sex-determining mechanisms in the androdioecious species *Datisca glomerata* and its dioecious sister species *D. cannabina*, using a combination of traditional genetic crosses and molecular markers. The use of molecular markers, along with an extensive set of genetic crosses, seemed particularly important in this study, because a previous crossing study in *D. glomerata* suggested that factors controlling sex ratios and/or sex determination might be complex (Wolf *et al.* 1997). The current study did not reveal such complexities.

In addition to providing basic information regarding the genetic control of a rare breeding system, comparisons between the two sister taxa can lead to insights concerning the sequence of reproductive-system evolution in Datisca, more accurate models of the evolution of androdioecy, and a better understanding of the long-term evolutionary stability of androdioecy.

MATERIALS AND METHODS

Study species: *D. glomerata* (Datiscaceae) is a tall, wind-pollinated, self-compatible, perennial angiosperm that occurs in riparian habitats throughout Baja California, Mexico, and California, USA (Davidson 1973). This species is one of the best studied examples of functional and morphological androdioecy in plants (Liston *et al.* 1989; Fritsch and Rieseberg 1992; Rieseberg *et al.* 1992). Males produce only male gametes, while hermaphrodites produce and gain fitness through both pollen and seed production. Further, males produce three times as much pollen as hermaphrodites (Philbrick and Rieseberg 1994; Spencer and Rieseberg 1995), outcrossing rates are high (65–92%; Fritsch and Rieseberg 1992), and the frequency of males is always <0.5 (0–0.31; Liston *et al.* 1990). In populations where selfing rates have been measured, the observed frequencies of males is consistent with that expected by theory, given the relative levels of pollen production and inbreeding depression observed in this species (Liston *et al.* 1990).

The only close relative of *D. glomerata* is *D. cannabina*

(Swensen *et al.* 1998), which is dioecious and found along streams and rocky hillsides in the eastern Mediterranean region and the Himalayas (Davidson 1973). Both *D. glomerata* and *D. cannabina* are diploid ($n = 11$; Sinoto 1929; Snow 1959) and appear to lack heteromorphic sex chromosomes (J. Qiu, L. H. Rieseberg and T. Philbrick, unpublished results, but see Sinoto 1929).

D. glomerata and *D. cannabina* are morphologically highly similar (Liston *et al.* 1989). Flowers in both species are apetalous, with short, inconspicuous calyx lobes and no nectaries (Davidson 1973). Male flowers in the two species are remarkably similar, lacking a gynoecium and bearing many anthers (11 ± 2.4 anthers in *D. glomerata*; Liston *et al.* 1990). Hermaphroditic flowers in *D. glomerata* consist of three linear, branched styles, a few anthers (3.0 ± 0.6; Liston *et al.* 1990), and a small (1 cm) capsule that produces 100–300 tiny (1 mm long) seeds. Except for a lack of anthers, female flowers in *D. cannabina* are nearly identical to the hermaphroditic flowers of *D. glomerata*. Phylogenetic (Rieseberg *et al.* 1992; Swensen *et al.* 1998) and theoretical (Charlesworth 1984, 1993) evidence suggests that androdioecy in the Datiscaceae may have evolved from dioecy. Thus, the genetics of sex determination in *D. cannabina* and the floral morphology of the species may represent the ancestral condition.

Genetic materials and crosses: Seeds were collected from three *D. glomerata* populations in southern California (USA): Baughman springs (BS), the San Juan Picnic Area (SJ; Wolf *et al.* 1997), and Cedar Springs Dam (CSD; Liston *et al.* 1990). Both BS and SJ populations had >500 plants, with male frequencies of 0.173 and 0.035, respectively, whereas the CSD population was composed of 23 hermaphrodite and 3 male plants.

Greenhouse-grown plants from two of these populations (BS and SJ) were used in a series of controlled crosses designed to investigate several unanswered questions from Wolf *et al.* (1997). In all, sex-ratio data were collected from 16 ☿ × ♂ and 32 ☿ × ☿ crosses, using six BS and two SJ males and eight BS and nine SJ hermaphrodites. Crosses were made on emasculated and bagged flowers to prevent pollen contamination. Offspring were germinated in a mist room and randomized before planting in the field or greenhouse to avoid effects from microenvironment variation.

Both within- and between-population crosses were made to examine interpopulation variation in sex-determining mechanisms. Additionally, these ☿ × ☿ crosses and ☿ × ♂ crosses were used to reveal genetic differences between males and hermaphrodites. Further, to examine the maternal (or cytoplasmic) influence on sex ratios, nine pairs of reciprocal ☿ × ☿ crosses were compared. Finally, we studied the possibility of an environmental influence on sex ratios using three analyses. First, sex ratios were compared between two environments (the Indiana University greenhouse and IU Botany Experimental Field), using four progeny arrays (two ☿ × ☿ and two ☿ × ♂ crosses) from which half of the progeny were grown in each environment. Second, sex ratios were compared between years, using six families that were planted in the field in both 1998 and 1999. Finally, to increase the sample size, sex-ratio data were pooled across all families within cross type (*i.e.*, ☿ × ☿ and ☿ × ♂) including the crosses above. Comparisons were made between the two years and between the two environments.

Because *D. cannabina* grows in the eastern Mediterranean and Himalayas and is difficult to collect, we obtained bulk-collected seeds from populations growing at two different botanical gardens: Botanischer Garten der Universität Bonn in Germany (G) and Conservatoire et Jardins Botaniques de Nancy in France (F). Seeds were bulk collected from the plants growing at these two gardens, but the number of parents founding the botanic-garden populations and the origin of those parents is unknown. Seeds from the botanic-garden collections were germinated and 40 plants from each collection were grown to maturity in the greenhouse. Females were isolated from males before flowers matured, and a total of 44 crosses, within and between the two collections, were carried out using seven females from each collection, seven males from the G collection, and four males from the F collection. Approximately 40 progeny per cross were grown to maturity and sexed.

Finally, interspecific crosses were made in all possible combinations. Because pollination success and seed viability were low, germination of all seeds was attempted. Flowering adults were genotyped with species-specific allozyme alleles of phosphoglucoisomerase and triosephosphate isomerase (Liston *et al.* 1989), and nonhybrids were discarded.

Statistics: Replicated *G*-tests were used to examine sex-ratio heterogeneity among crosses and to assess goodness of fit to 1:1 sex ratios (Sokal and Rohlf 1995). When cells contained less than five counts, Fisher's exact test was used to examine heterogeneity among crosses, and goodness of fit was assessed by calculating the probability of the observed sex ratio or any more extreme sex ratio. Hierarchical log-linear analyses (logit model *sensu* Goodman 1972) were used to examine environmental effects on sex ratios and to determine whether there were differences between populations. In these analyses, sex ratio (number of males/hermaphrodites) was a conceptualized dependent variable and other factors such as environment and year were considered to be independent variables. In the base model, the independent variables were assumed to have no influence on sex ratios, while in the saturated model, all factors and interactions influenced sex ratios (*i.e.*, they were all included in the model). To compare the fit of various models to the data, the difference between the likelihood ratio statistics (L^2; calculated with SAS PROC CATMOD) of each model was compared to the χ^2 distribution. If the saturated model did not fit the data significantly better ($P < 0.05$) than the base model, we concluded that none of the factors had a significant influence on sex ratios. Otherwise, factors (and interaction terms) were sequentially added to the base model to determine which factor(s) influenced sex ratios and significantly improved the model; new models were compared to the model one step lower in the hierarchy.

Laboratory methods: Total genomic DNA was extracted from dried or frozen leaf tissue using the QIAGEN (Valencia, CA) DNeasy plant mini kit. Amplified fragment length polymorphism (AFLP; Vos *et al.* 1995) genotyping followed the protocol described in Noyes and Rieseberg (2000), with some modifications. Whole genomic DNA (150 ng) was simultaneously digested with 3 units each *Eco*RI and *Mse*I for 3 hr at 37°. Adapter ligation was carried out using 10 units T4 ligase at 20° for an additional 3 hr. Preamplification was performed with *Eco*RI and *Mse*I cut-site primers (Ea and Ma, respectively), each having one selective nucleotide (adenosine). Reactions were carried out in 25 μl volume with 2 μl of the ligation mix, PCR buffer as described by Keim *et al.* (1997), 0.1875 μg each primer, 1.5 mM Mg^{2+}, 0.04 mM each dNTP, and 1 unit Taq polymerase. The PCR product was diluted 1:19 in 1 × TE, and 2.5 μl was used in the 10-μl selective amplification. Primers for the selective amplification had an additional 2–4 selective nucleotides, which are indicated after an E for *Eco*RI or an M for the *Mse*I primer, and an a for the selective nucleotide used in preamplification (*e.g.*, Eact/Magg for a pair of primers with CT and GG, respectively, in the selective amplification). Chemical conditions in the selective amplifications were the same as for the preamplifications, except that 5 ng of the fluorescently labeled *Eco*RI primer was used with 15 ng of the *Mse*I primer. PCR products were separated on 5% polyacryl-

amide gels and visualized with a Hitachi FMBIOII fluorescent imager (Hitachi Software Engineering, Tokyo). The presence or absence of fragments was scored manually.

Linkage between AFLPs and sex expression was analyzed in one family from each species. The two families were produced by crosses between BS-34C.1☿ × SJ-4.1♂ in *D. glomerata* (notation described in Table 2) and F1♀ × G1♂ in *D. cannabina*. In each species, initial screening of AFLP primer pairs was done on eight full sibs, four of each sex. After potentially sex-linked markers were identified, all remaining siblings and their parents were genotyped to estimate the centimorgan (cM) distance between the putative sex-determining locus and the marker. If the marker was closely linked to sex, additional conspecific, interspecific, and hybrid individuals were genotyped to examine linkage disequilibrium between sex and the identified marker.

Because no markers were perfectly associated with sex in the dioecious species (*D. cannabina*), linkage mapping was used to examine the genetic architecture of sex determination.

Linkage mapping: After potentially sex-linked markers were identified in the *D. cannabina* F1♀ × G1♂ family, a linkage map was made by genotyping 29 female and 32 male offspring with the 13 primer pairs exhibiting sex-linked fragments (Figure 2). Eighty unambiguous and easily scored bands were present in the sire, absent in the dam, and showed segregation in the progeny, suggesting that the sire was heterozygous (+ −) and the dam was homozygous (− −) at the loci producing the bands. Only 14 loci appeared to be heterozygous (+ −) in the dam and homozygous (− −) in the sire. Therefore, only the male parent's genome was mapped. The linkage map was constructed with MAPMAKER/EXP 3.0 (Lander *et al.* 1987), using the BC1 design, the Kosambi mapping function, and a LOD threshold of 3.0. Linked repulsion-phase markers were mapped by entering each dominant marker as scored (*i.e.*, band present, +; band absent, −) and its inverse (*i.e.*, band present, −; band absent, +).

RESULTS

Molecular markers: A total of 191 scorable, polymorphic markers were identified with 112 AFLP primer pairs in *D. glomerata*, while 2110 polymorphic markers were found with 250 primer pairs in *D. cannabina*.

Markers, androdioecious *D. glomerata*: Two sex-linked markers were found in this species. A 60-bp marker was amplified with the Eact/Magg primer pair in 6 out of 24 males, 13 out of 16 hermaphrodites, and the sire (SJ-4.1♂) of the BS-34C.1☿ × SJ-4.1♂ family. Because the marker was found in the male parent, but not the hermaphrodite parent, these results suggest that the male parent is heterozygous for the sex-determining locus. Because the marker was transmitted primarily to hermaphrodite offspring, it appears to be ~22.5 cM away from a recessive hermaphrodite-determining allele at the sex-determining locus. Unsurprisingly, the marker did not show widespread linkage disequilibrium with sex, but was associated with sex only in the BS-34C.1☿ × SJ-4.1♂ family.

A second marker provides much stronger support for the hypothesis of dominant maleness at a single sex-determining locus. The second marker was tightly associated with sex in all genotyped individuals (Figure 1). The 280-bp marker was originally amplified with Eaac/Maca but, by adding selective bases to the Mse primer, was found to also amplify with Eaac/Macaac. The marker amplified in all 25 male siblings and their sire (SJ-4.1♂) and failed to amplify in the 19 hermaphrodite siblings and their dam (BS-34C.1☿). Thus, the marker appears to be heterozygous in the male parent and linked to the male-determining allele. The marker was likewise sex linked in all tested individuals from the other populations (4 males and 8 hermaphrodites from the SJ population, 11 males and 4 hermaphrodites from BS, and 3 males and 5 hermaphrodites from CSD, all of which were either wild collected or descended from different wild-collected plants). This suggests that sex in *D. glomerata* is determined by a single segregating locus at which males are heterozygous and hermaphrodites are homozygous (*i.e.*, the male-determining allele is dominant to the hermaphrodite-determining allele). Additionally, because linkage disequilibrium between sex and the marker is widespread over at least three populations separated by ~650 km and the San Gabriel Mountains, the marker locus is likely to be either extremely close to the sex-determining locus or in a region of reduced recombination around the sex-determining locus.

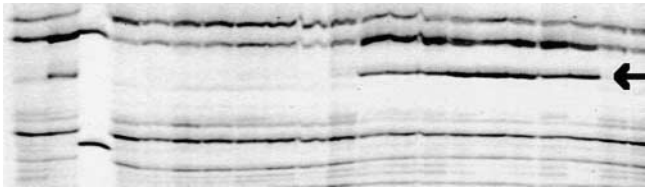

Figure 1.—Sex-linked AFLP marker (arrow) present in males and absent in hermaphrodites of the androdioecious *D. glomerata*. Shown from left to right are the hermaphroditic maternal parent (BS 34C.1), the male parent (SJ 4.1), ladder (300-bp and 250-bp fragments), eight hermaphroditic offspring, eight male offspring, and two more hermaphroditic offspring. The 280-bp fragment was amplified with the Eaac/Macaac primer pair and run here on a 12% polyacrylamide gel (Sambrook *et al.* 1989).

We also used the Eaac/Macaac primer pair to genotype individuals of the dioecious species and hybrids. The marker was completely absent in both sexes of *D. cannibina*. However, it was present in males of *D. cannabina*♀ × *D. glomerata*♂ interspecific hybrids and absent in the females (total of 4 ♂, 12 ♀ offspring from five different crosses). This suggests that sex is controlled by the same locus in hybrids as in *D. glomerata* and therefore may be controlled by homologous loci in both parental species.

Markers, dioecious *D. cannibina*: Seventy of the 80 loci mapped to 11 linkage groups, corresponding to $n = 11$ chromosomes (Sinoto 1929; Snow 1959). Linkage groups ranged in size from 2 to 11 markers (average = 6.3), with a total map length of 348 cM (average = 31.6

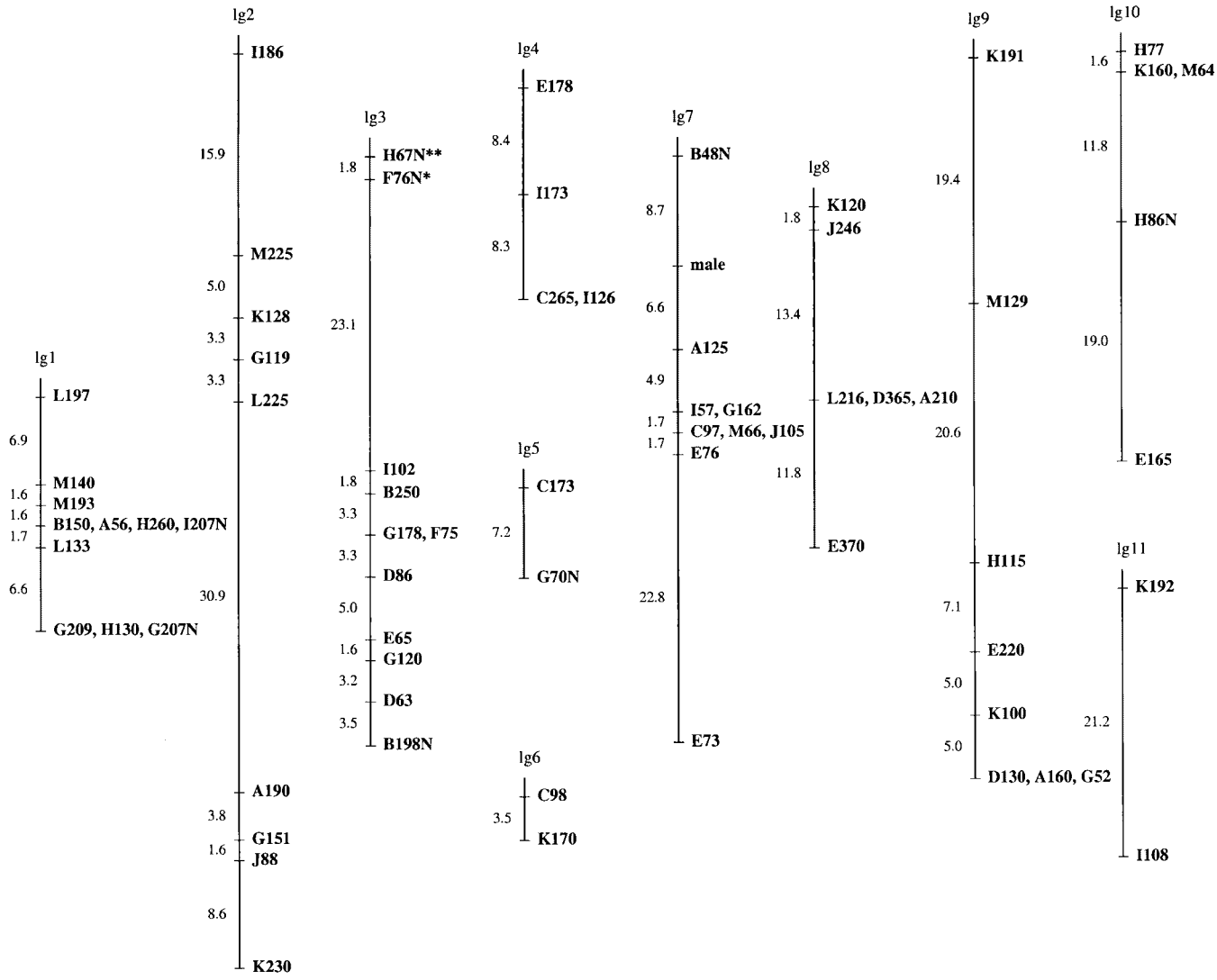

FIGURE 2.—Genetic linkage map of AFLP markers in dioecious *D. cannibina*, with the sex-determining locus on lg7. Markers are indicated on the right of each linkage group, and the genetic distance between each marker (Kosambi cM) is on the left. Loci <1 cM apart are separated by commas. Asterisks denote loci that deviate from Mendelian inheritance (experimentwise $*P \leq 0.05$; $**P \leq 0.01$). Markers are represented by the primer code letter the size of the mapped fragment (bp) and N when the locus was linked in repulsion phase to the other mapped loci. Primer code letter A represents the primer pair Eaac-Matag; B, Eaac-Mattc; C, Eacc-Matt; D, Eacg-Magt; E, Eact-Maggc; F, Eact-Matagc; G, Eact-Matc; H, Eata-Matagg; I, Eatg-Macaa; J, Eatg-Magt; K, Eatg-Mattc; L, Eattc-Maac; M, Eattc-Maag.

cM per linkage group). On average, linked markers were separated by 5.0 cM. However, markers were not uniformly distributed, clustering around areas that likely correspond to centromeres (VUYLSTEKE *et al.* 1999; YOUNG *et al.* 1999). Only 2 loci showed significant deviations from the expected 1:1 segregation ratios, and these did not map to the linkage group involved in sex determination (lg7).

None of the primer pairs amplified markers that were perfectly associated with sex in *D. cannabina*, but eight markers showed loose sex linkage (within 10 cM). All of these markers were closely linked to each other on one linkage group (lg7; Figure 2), and lg7 was the only linkage group with which sex showed any association. Further, all of the sex-linked markers were present in the sire (G1♂) and absent in the dam (F1♀) of the examined cross. These data suggest that males are heterozygous and females are homozygous at a single sex-determining locus (or a few closely linked loci) and that maleness is dominant to femaleness.

Since recombination is often reduced around sex-determining loci (*e.g.*, CHARLESWORTH and CHARLESWORTH 1978; BULL 1983; YI and CHARLESWORTH 2000), we might expect to see markers clustering around the sex-determining locus, even if the sex chromosomes have not diverged enough to show morphological differences (CHARLESWORTH and CHARLESWORTH 1978; BULL 1983). Although the initial screening for sex-linked

TABLE 1

Progeny resulting from each type of cross, pooled across families

Cross type	Progeny ♂	Progeny ♀	Progeny ☿	Progeny NF	No. of crosses	Heterogeneity d.f.	Heterogeneity G_H	Pooled d.f.	Pooled G_P
dc G♀ × dc G♂	253	321		NA	17	16	10.58	1	8.07**
dc F♀ × dc G♂	148	151		NA	6	5	5.31	1	0.03
dc F♀ × dc F♂	49	769		NA	19	$P = 0.34$		1	763.1****
dc G♀ × dc F♂	1	19		NA	2	$P = 1.00$		$P = 0.00004$****	
dg☿ × dg☿	13		1152	505	31	$P = 0.19$		1	1471.7****
dg☿ × dg♂	407		414	288	16	15	20.2	1	0.06
dc♀ × dg☿	8	195		190	13	$P = 0.23$		1	213.5****
dc♀ × dg♂	100	113		134	12	11	2.70	1	0.79
dg☿ × dc♂	3	10		15	6	$P = 1.00$		$P = 0.092$	

dc, *D. cannabina* (dioecious); dg, *D. glomerata* (androdioecious); F, French collection; G, German collection; NF, individuals not flowering; NA, nearly all *D. cannabina* plants flowered (>90%), but numbers are not available. Replicated goodness of fit tests (to a 1:1 sex ratio; *G*-statistic) were used to calculate *G* for heterogeneity and the pooled *G* (fit to a 1:1 ratio). Total *G* and partitioned *G* values are in Tables 2–6. Some crosses produced too few males for a replicated *G*-test ($N < 5$), so a Fisher's exact test, which yields only a *P* value, was used to test for heterogeneity among crosses, and crosses were pooled to calculated *G* for goodness of fit. When cell sizes were still too small for a goodness-of-fit *G*-test after pooling families, the probability of the data or any more extreme data was calculated from a binomial distribution. *$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$0.0001 < P < 0.001$; ****$P < 0.0001$.

markers was extensive (2110 polymorphic loci), no markers perfectly cosegregated with sex. Instead, there were six tightly linked markers clustered ~10 cM from the sex-determining locus. There are several possible explanations for the lack of a perfectly sex-linked marker and the nearby cluster of markers. First, it is possible that sex was scored incorrectly in the three individuals showing "recombination" (*i.e.*, when the marker was associated with the unexpected sex) and that several of the markers are actually 0 cM from the sex-determining locus. Another possibility is that several closely linked loci on lg7 are involved in sex determination, preventing us from finding any perfectly sex-linked markers; it is difficult to distinguish between a single locus and several tightly (but not perfectly) linked loci. On the other hand, our inability to find an AFLP locus perfectly linked to the sex-determining locus may simply be due to chance. The cluster of markers could simply correspond to the centromere, where recombination is often suppressed (Copenhaver *et al.* 1999), in which case the sex-determining locus must be ~10 cM from the centromere.

Crossing data, androdioecious *D. glomerata*: Hermaphrodite × male crosses produced both male and hermaphrodite offspring at approximately equal frequencies, and ☿ × ☿ crosses produced highly hermaphrodite-biased sex ratios, with almost no male offspring (Tables 1, 2, and 3). Additionally, there was no significant heterogeneity among ☿ × ♂ crosses or among ☿ × ☿ crosses (*i.e.*, crosses with fathers of the same sex all produced similar sex ratios; Table 1). Thus, the data are generally consistent with a single-locus model, in which maleness is dominant. Although male offspring are not expected in ☿ × ☿ crosses under the single-locus model, the small number of males produced (13 males out of 1165 offspring) are most likely due to pollen or seed contamination. An alternative explanation is that males are frequently generated through some type of recurrent mutation. *C. elegans* hemaphrodites spontaneously produce males through selfing at nearly the same order of magnitude (0.2% male offspring; Hodgkin and Doniach 1997). Additionally, many studies of sex determination find crosses that produce a small number of progeny with the unexpected sex (*e.g.*, Pannell 1997b; Charlesworth and Laporte 1998; Dudle *et al.* 2001). However, it would be surprising to find a marker showing a widespread disequilibrium with sex expression (above) if males were frequently being generated through mutation in *D. glomerata*.

In a previous study, more complex sex ratios were observed (Wolf *et al.* 1997), suggesting that sex determination might be influenced by an additional allele or locus at which hermaphroditism is dominant, a cytoplasmic (or maternal) factor, and/or genetic differences among populations. However, as described next, our investigations failed to provide evidence for these complexities (Table 4).

To determine if there were sex-ratio differences between the SJ and BS populations, we used a three-way log-linear analysis for each cross type (☿ × ☿ and ☿ × ♂), which included maternal population, paternal population, and progeny sex as factors. For both cross types (Tables 2 and 3), the base model fit the data well ($L^2_{\text{d.f.}=3} = 2.75$, $P = 0.43$ for ☿ × ☿ crosses and $L^2_{\text{d.f.}=3} =$

TABLE 2

Sex ratios from androdioecious *D. glomerata* hermaphrodite × hermaphrodite crosses

Mother		Father	Progeny ♂	Progeny ⚥	Progeny Not flowering	*P* value
Reciprocal crosses						
BS-1.1⚥	×	BS-16.1⚥	0	40	38	$P = 1.0$
BS-16.1⚥	×	BS-1.1⚥	0	45	17	
BS-1.1⚥	×	BS-9C.1⚥	1	40	25	$P = 1.0$
BS-9C.1⚥	×	BS-1.1⚥	0	37	22	
BS-30.1⚥	×	BS-9C.1⚥	0	23	10	$P = 1.0$
BS-9C.1⚥	×	BS-30.1⚥	0	11	6	
SJ-7.1⚥	×	SJ-4.1⚥	0	43	34	$P = 1.0$
SJ-4.1⚥	×	SJ-7.1⚥	0	33	16	
SJ-4.1⚥	×	SJ-21.1⚥	0	75	30	$P = 1.0$
SJ-21.1⚥	×	SJ-4.1⚥	0	54	37	
SJ-4.1⚥	×	SJ-25.1⚥	0	69	33	$P = 2.0$
SJ-25.1⚥	×	SJ-4.1⚥	2	55	24	
SJ-4.1⚥	×	BS-9C.1⚥	5	68	30	$P = 0.15$
BS-9C.1⚥	×	SJ-4.1⚥	0	46	19	
SJ-11.1⚥	×	BS-1.1⚥	0	64	27	$P = 1.0$
BS-1.1⚥	×	SJ-11.1⚥	1	75	37	
SJ-15.1⚥	×	BS-1.1⚥	0	57	34	$P = 1.0$
BS-1.1⚥	×	SJ-15.1⚥	1	67	19	
Nonreciprocal crosses						
BS-1.1⚥	×	SJ-25.1⚥	0	17	0	
BS-19.1⚥	×	SJ-4.1⚥	0	24	6	
BS-30.1⚥	×	SJ-15.1⚥	1	23	1	
BS-9C.1⚥	×	SJ-11.1⚥	0	12	3	
BS-9C.1⚥	×	SJ-15.1⚥	0	14	2	
SJ-15.1⚥	×	BS-30.1⚥	0	20	2	
SJ-21.1⚥	×	SJ-15.1⚥	0	22	11	
SJ-4.1⚥	×	BS-1.1⚥	1	22	8	
SJ-4.1⚥	×	BS-11.1⚥	0	2	0	
SJ-4.1⚥	×	BS-11A.1⚥	0	23	2	
SJ-4.1⚥	×	SJ-11.1⚥	0	12	3	
SJ-4.1⚥	×	SJ-15.1⚥	0	20	5	
SJ-4.1⚥	×	SJ-4.1⚥	0	21	4	
SJ-4.1⚥	×	SJ-5.1⚥	1	18	0	
Total			13	1152	505	

P values are from pairwise Fisher's exact tests for differences between reciprocal crosses. No *P* values are included for crosses performed in only one direction. Notation describing parents: Letters (SJ or BS) represent the population of origin. Numbers before the decimal differentiate wild plants from which seeds were collected. Numbers after the decimal differentiate offspring of each wild plant. For example, SJ-4.1⚥ is hermaphroditic offspring number 1 from wild plant number 4 in the SJ population.

1.20, $P = 0.75$ for ⚥ × ♂ crosses), suggesting that the sex ratios are not influenced by the population from which either the maternal or the paternal parent originated.

Cytoplasmic (maternal) influence on sex ratios was examined through pairwise comparisons of reciprocal ⚥ × ⚥ crosses. There were no significant differences (Table 2), suggesting a lack of maternal influence. However, given the very small number of males resulting from ⚥ × ⚥ crosses, these statistics may not be extremely informative. We did not explicitly investigate the possibility of a maternal influence on ⚥ × ♂ crosses. However, the lack of significant heterogeneity among ⚥ × ♂ crosses overall (Table 1) and the lack of a difference between populations (above) fail to provide any reason to suspect the existence of maternally inherited variation that influences sex ratios or sex expression.

Finally, we examined the possibility of an environmental component to sex or sex-ratio determination by comparing the sex ratios of plants growing in two locations (field *vs.* greenhouse) in 2 consecutive years

TABLE 3

Sex ratios of androdioecious *D. glomerata* hermaphrodite × male crosses

			Progeny			Goodness of fit (1:1)	
Mother		Father	♂	☿♂	Not flowering	d.f.	*G*
BS-1.1☿♂	×	BS-36.2♂	28	30	47	1	0.069
BS-1.1☿♂	×	SJ-7.1♂	27	30	28	1	0.16
BS-34C.1☿♂	×	SJ-4.1♂	28	30	8	1	0.069
BS-9C.1☿♂	×	BS-12.1♂	36	41	54	1	0.32
SJ-11.1☿♂	×	BS-7.2♂	6	5	1	1	0.091
SJ-11.1☿♂	×	SJ-4.1♂	12	8	5	1	0.81
SJ-23.1☿♂	×	SJ-7.1♂	6	16	6	1	4.72*
SJ-24.1☿♂	×	SJ-4.1♂	13	10	2	1	0.39
SJ-24.1☿♂	×	SJ-7.1♂	14	5	4	1	4.44*
SJ-25.1☿♂	×	SJ-4.1♂	9	16	3	1	1.99
SJ-4.1☿♂	×	BS-11.1♂	48	42	26	1	0.40
SJ-4.1☿♂	×	BS-12.1♂	33	38	14	1	0.35
SJ-4.1☿♂	×	BS-36.1♂	30	37	17	1	0.73
SJ-4.1☿♂	×	BS-9C.1♂	22	24	11	1	0.087
SJ-5.1☿♂	×	SJ-4.1♂	39	47	30	1	0.75
SJ-5.1☿♂	×	SJ-7.1♂	56	35	32	1	4.89*
Total			407	414	288	16	20.26

Since sex ratios were not significantly heterogeneous, nor did the pooled sex ratio differ from 1:1 (Table 1), partitioned *G* values that are significant with a testwise $\alpha = 0.05$ are not more extreme than expected by chance. * Testwise $0.01 < P < 0.05$.

(Table 4). Field conditions in 1998 and 1999 were apparently quite different. In 1999, plants grew vigorously and required no watering, whereas the spring and early summer of 1999 were dry; plants required irrigation, and many went dormant or died before flowering (Table 4). When pooling across all families within a cross type, three-way log-linear analyses revealed no significant effect of year or location on sex ratios in either cross type; the models that excluded the effects of year and location fit the observed data with a high likelihood ($L^2_{\text{d.f.}=3} = 2.16$, $P = 0.54$ for ☿♂ × ☿♂ crosses and $L^2_{\text{d.f.}=3} = 3.20$, $P = 0.36$ for ☿♂ × ♂ crosses).

A separate log-linear analysis of the six ☿♂ × ♂ families that were grown in the field in both 1998 and 1999, in which family was included as a factor, also failed to reveal significant differences between years ($\Delta L^2 = 1.67$, d.f. = 1, $P = 0.20$). However, there was a marginally significant effect of family on sex ratios ($\Delta L^2 = 10.31$, d.f. = 5, $P = 0.07$). This effect disappeared when the male-biased SJ-5.1☿♂ × SJ-7.1♂ family was removed from the analysis; the base model fit the data well ($L^2_{\text{d.f.}=9} = 7.57$, $P = 0.58$), suggesting that this family may be behaving differently from other families. Differences in ☿♂ × ☿♂ crosses were not analyzed because very few males were produced (Table 4).

A similar analysis was used to examine the effects of location (field *vs.* greenhouse) on within-family sex ratios in 1999. Neither of the two ☿♂ × ☿♂ crosses produced any males (Table 4), so only the two ☿♂ × ♂ crosses were analyzed. There was no significant effect of family ($\Delta L^2 = 2.72$, d.f. = 1, $P = 0.099$), but there was a significant effect of location ($\Delta L^2 = 9.26$, d.f. = 1, $P = 0.002$). This effect is again caused by the SJ-5.1☿♂ × SJ-7.1♂ family. It produced a male-biased sex ratio in the field ($P = 0.0015$), but no bias in the greenhouse ($G_{\text{d.f.}=1} = 0.082$, $P = 0.77$). Although the results from this family could be viewed as evidence for environmental sex determination, the sex-linked marker opposes this interpretation. In 1999, 98% of the greenhouse-grown plants in this family flowered, whereas only 41% of field-grown plants flowered. Thus, it is likely that males from this family flowered at a rate higher than that of hermaphrodites, producing the male-biased sex ratios when plants were grown in the field. If all plants had flowered, as in the greenhouse, sex ratios would likely be 1:1. This interpretation is consistent with data indicating that *D. glomerata* males flower earlier than hermaphrodites in their natural habitat (Spencer and Rieseberg 1995).

Crossing data, dioecious *D. cannabina*: Unlike the androdioecious species, sex ratios in the dioecious species were influenced by the genetic stock of the parents—more specifically, by the collection of the father. After receiving bulk-collected seeds, 40 plants from each botanic garden were grown to adulthood. The sex ratio of the G collection was not different from 1:1 (15 ♀: 14♂; $G_{\text{d.f.}=1} = 0.034$, $P = 0.85$), but the F collection was highly female biased (24♀: 4♂; $P = 0.0002$). Further, in the next generation, crosses with F fathers all produced highly female-biased sex ratios, whereas crosses with G

TABLE 4

Sex ratios from androdioecious *D. glomerata* partitioned according to year and location

Mother		Father	Field ♂	Field ☿♂	Field Not flowering	Greenhouse ♂	Greenhouse ☿♂	Greenhouse Not flowering
			Pooled 1998					
dg☿♂	×	dg♂	104	111	39	24	26	NA
dg☿♂	×	dg☿♂	5	290	80	1	146	NA
			Pooled 1999					
dg☿♂	×	dg♂	185	166	248	91	111	NA
dg☿♂	×	dg☿♂	6	502	425	1	215	NA
			By family 1999					
BS1.1☿♂	×	SJ-15.1☿♂	0	12	16	0	35	5
SJ-4.1☿♂	×	SJ-21.1☿♂	0	14	29	0	40	0
SJ-5.1☿♂	×	SJ-7.1♂	18	3	30	20	19	1
BS1.1☿♂	×	BS36.2♂	12	7	47	16	23	1

Mother		Father	1998 ♂	1998 ☿♂	1998 Not flowering	1999 ♂	1999 ☿♂	1999 Not flowering
			By family in the field					
SJ-4.1☿♂	×	BS-11.1♂	6	9	4	39	25	22
SJ-4.1☿♂	×	BS-12.1♂	4	8	3	21	24	11
SJ-4.1☿♂	×	BS-36.1♂	9	12	1	19	18	16
SJ-4.1☿♂	×	BS-9C.1♂	3	4	2	11	9	9
SJ-5.1☿♂	×	SJ-4.1♂	8	6	4	27	36	25
SJ-5.1☿♂	×	SJ-7.1♂	10	8	2	18	3	30
BS-1.1☿♂	×	SJ-11.1☿♂	0	12	3	1	37	34
BS-1.1☿♂	×	SJ-15.1☿♂	0	9	3	0	12	16
BS-9C.1☿♂	×	SJ-4.1☿♂	0	17	8	0	22	11
SJ-11.1☿♂	×	BS-1.1☿♂	0	12	5	0	41	22
SJ-15.1☿♂	×	BS-1.1☿♂	0	14	5	0	23	29
SJ-25.1☿♂	×	SJ-4.1☿♂	2	14	5	0	37	19
SJ-4.1☿♂	×	BS-9C.1☿♂	0	14	2	4	42	28
SJ-4.1☿♂	×	SJ-21.1☿♂	0	21	1	0	14	29
SJ-4.1☿♂	×	SJ-25.2☿♂	0	16	1	0	41	32

NA, data not available.

fathers produced 1:1 sex ratios or a slight excess of females (Tables 1 and 5). There was no heterogeneity within cross types (*i.e.*, G♀ × G♂, F♀ × F♂, F♀ × G♂, and G♀ × F♂; Table 1). Thus, we went on to examine the statistical influence of maternal and paternal collection on sex ratios. Log-linear analysis revealed that although the base model did not fit the data well ($L^2_{\text{d.f.}=3} = 392.78$, $P < 0.0001$), a model including paternal collection as a factor significantly improved the fit ($L^2_{\text{d.f.}=1} = 390.42$; $P > 0.0001$) and was not significantly worse than the saturated model ($L^2_{\text{d.f.}=2} = 2.36$, $P > 0.3$). Adding the maternal collection to the model did not significantly improve the fit after including the paternal collection ($L^2_{\text{d.f.}=1} = 2.36$, $P > 0.1$). Thus, we conclude that the father's collection had an influence on sex ratios, but the mother's collection apparently did not.

Under a single-locus genetic model of sex determination, one would generally expect 1:1 sex ratios from all crosses. However, the lack of sex-ratio heterogeneity within cross type and the consistency of sex ratios over two generations are not consistent with a multilocus model. Taken with the molecular marker data, these crossing data suggest that there is a single sex-determining locus in the individuals studied and that sex ratios are biased by some other factor, such as meiotic drive or a cytoplasmic factor.

Interspecific hybrids: Similar to the *D. glomerata* crosses, hybrid crosses using hermaphrodites as pollen donors produced very few males, whereas crosses using male pollen donors produced 1:1 sex ratios (Tables 1 and 6). Further, there was no heterogeneity within cross-types (Tables 1 and 6). These sex ratios are consistent

TABLE 5

Sex ratios from dioecious *D. cannabina*

Mother		Father	Progeny ♂	Progeny ♀	Goodness of fit (1:1) d.f.	Goodness of fit (1:1) *G*
G1♀	×	G1♂	5	3	1	0.51
G1♀	×	G3♂	4	7	1	0.83
G1♀	×	G4♂	20	20	1	0
G2♀	×	G1♂	19	19	1	0
G2♀	×	G4♂	8	10	1	0.22
G2♀	×	G6♂	11	17	1	1.30
G3♀	×	G1♂	20	20	1	0
G3♀	×	G3♂	11	21	1	3.18
G4♀	×	G1♂	15	25	1	2.53
G4♀	×	G4♂	22	18	1	0.40
G4♀	×	G6♂	12	16	1	0.57
G5♀	×	G1♂	12	23	1	3.52
G5♀	×	G5♂	18	18	1	0
G5♀	×	G6♂	5	10	1	1.70
G7♀	×	G1♂	22	34	1	2.60
G7♀	×	G6♂	15	16	1	0.03
G7♀	×	G7♂	34	44	1	1.29
Total			253	321	17	18.66
F1♀	×	G1♂	32	29	1	0.15
F1♀	×	G2♂	16	25	1	1.99
F1♀	×	G6♂	29	20	1	1.66
F1♀	×	G7♂	24	24	1	0
F2♀	×	G2♂	27	36	1	1.29
F2♀	×	G7♂	20	17	1	0.24
Total			148	151	6	5.34
F1♀	×	F1♂	4	41	$P = 9.3 \times 10^{-9}$	
F1♀	×	F2♂	3	32	$P = 4.2 \times 10^{-7}$	
F1♀	×	F3♂	1	24	$P = 1.5 \times 10^{-6}$	
F2♀	×	F1♂	6	47	$P = 5.8 \times 10^{-9}$	
F2♀	×	F2♂	3	35	$P = 1.9 \times 10^{-8}$	
F2♀	×	F3♂	0	42	$P = 4.5 \times 10^{-19}$	
F3♀	×	F1♂	5	40	$P = 7.8 \times 10^{-8}$	
F3♀	×	F3♂	2	34	$P = 1.9 \times 10^{-8}$	
F4♀	×	F1♂	2	57	$P = 6.1 \times 10^{-15}$	
F4♀	×	F2♂	1	31	$P = 1.5 \times 10^{-8}$	
F4♀	×	F3♂	5	35	$P = 1.3 \times 10^{-6}$	
F5♀	×	F1♂	1	39	$P = 7.5 \times 10^{-11}$	
F5♀	×	F2♂	4	54	$P = 3.2 \times 10^{-12}$	
F5♀	×	F3♂	2	40	$P = 4.1 \times 10^{-10}$	
F6♀	×	F1♂	3	54	$P = 4.3 \times 10^{-13}$	
F6♀	×	F2♂	3	33	$P = 2.3 \times 10^{-7}$	
F6♀	×	F3♂	0	46	$P = 2.8 \times 10^{-14}$	
F7♀	×	F1♂	1	42	$P = 1.0 \times 10^{-11}$	
F7♀	×	F3♂	3	43	$P = 4.6 \times 10^{-10}$	
Total			49	769	$P = 2.5 \times 10^{-167}$	
G1♀	×	F2♂	1	11	$P = 0.006$	
G1♀	×	F3♂	0	8	$P = 0.008$	
Total			1	19	$P = 4.0 \times 10^{-5}$	

G values for each cross represent partitions of the total *G* from the replicated *G*-test for which the pooled *G* and heterogeneity *G* are presented in Table 1. None of these tests were significant. When cell sizes were too small for *G*-tests, the probability of the data or any more extreme data was calculated from a binomial distribution. To apply a Bonferroni correction, *P* values can be divided by 19 for F♀ × G♂ crosses and by 2 for G♀ × F♂ crosses. All of the direct tests indicate significant differences from 1:1 sex ratios, even after a Bonferroni correction.

with a single segregating sex-determining locus in hybrids, at which maleness is dominant, if we assume that in the ♀ × ⚥ crosses, males were the product of contamination. These data would then support the hypothesis of a single male-dominant sex-determining locus in each parental species. Additionally, the crossing results, along with the observation that the *D. glomerata* sex-linked marker cosegregated with sex in hybrids, suggest that the same segregating locus determines sex in both *D. glomerata* and in the hybrids, and therefore, the segregating sex-determining loci in the two species are likely to be homologous.

A second, unexpected finding was that, although many of the crosses used hermaphroditic pollen or ovule donors, no hermaphroditic offspring were produced: only males and females. Because these crosses included dc♀ × dg♂ crosses with the dc cytotype, and dg⚥ × dc♂ crosses with the dg cytotype, the lack of hermaphrodites is clearly not due to cytoplasmic differences between species. Rather, the genetic element differentiating females from hermaphrodites must be a nuclear locus at which femaleness is dominant to hermaphroditism (male fertility is recessive).

The genetic factor differentiating females from hermaphrodites, however, may not be the same as that differentiating males from females and hermaphrodites (the primary sex-determining locus). Hermaphrodites may carry a new allele at or linked to the primary sex-determining locus, or there may be an unlinked mutation that confers male fertility to females and is fixed in the androdioecious population. To determine if the male-fertility factor was physically linked to the primary sex-determining locus, 50–100 F_2 crosses were attempted. Unfortunately, no viable seeds were obtained, presumably due to low F_1 pollen viability. [Viability was examined in 500–1000 pollen grains per donor, using 14 F_1 donors and 16 nonhybrid donors. The stain consisted of 30% sucrose and 0.1% MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). Only 0.06 ± 0.08 of F_1 pollen was fully or partially stained, whereas 0.57 ± 0.19 of nonhybrid pollen was fully stained and none was partially stained.] A similar number of backcrosses was attempted, but only one plant was obtained (a male).

DISCUSSION

Sex determination: The main focus of this article is the genetic basis of sex determination in the androdioecious *D. glomerata* and its dioecious sister species *D. cannabina*. Sex appears to be genetically determined by a single locus at which maleness is dominant in both species. Additionally, hermaphroditism is recessive to femaleness in interspecific crosses, suggesting that if androdioecy arose from dioecy, hermaphroditism arose through a recessive mutation. Our findings conflict with an earlier study in *D. glomerata* (Wolf *et al.* 1997) in

TABLE 6

Sex ratios from interspecific crosses

Mother		Father	Progeny ♂	Progeny ♀	Progeny Not flowering	Goodness of fit (1:1) d.f.	Goodness of fit (1:1) *G*
D. cannabina♀ × *D. glomerata*♂							
F3♀	×	BS-11.1♂	4	4	0	1	0
F4♀	×	SJ-4.1♂	28	32	54	1	0.27
F4♀	×	SJ-7.1♂	3	3	8	1	0
F6♀	×	BS-11.1♂	10	11	5	1	0.048
F6♀	×	BS-36.2♂	13	14	14	1	0.037
F6♀	×	SJ-7.1♂	4	4	3	1	0
G3♀	×	SJ-4.1♂	4	5	1	1	0.11
G5♀	×	SJ-4.1♂	6	4	0	1	0.40
G6♀	×	BS-7.2♂	8	7	12	1	0.067
G6♀	×	SJ-7.1♂	8	8	22	1	0
G7♀	×	BS-9C.1♂	4	8	15	1	1.36
G5♀	×	SJ-1B♂	8	13	0	1	1.2
Total			100	113	134	12	3.15
D. cannabina♀ × *D. glomerata*⚥							
F2♀	×	SJ-5.1⚥	0	4	2		
F4♀	×	SJ-25.1⚥	1	11	4		
F4♀	×	SJ-4.1⚥	4	60	31		
F4♀	×	SJ-5.1⚥	0	11	4		
F4♀	×	SJ-7.1⚥	0	41	69		
F6♀	×	BS-6C.2⚥	0	13	10		
G3♀	×	SJ-21.1⚥	0	11	1		
G5♀	×	SJ-21.1⚥	0	13	42		
G6♀	×	BS-1.1⚥	0	14	11		
G7♀	×	BS-1.1⚥	1	5	1		
G7♀	×	BS-9C.1⚥	0	1	6		
G7♀	×	SJ-23.1⚥	0	2	1		
G7♀	×	SJ-4.1⚥	2	9	8		
Total			8	195	190		
D. glomerata⚥ × *D. cannabina*♂							
BS-1.1⚥	×	F3♂	1	3	3		
BS-1.1⚥	×	F4♂	0	1	1		
BS-1.1⚥	×	F6F2-A♂	1	2	4		
SJ-15.1⚥	×	G7G6-B♂	0	1	6		
SJ-4.1⚥	×	F2♂	1	1	1		
SJ-4.1⚥	×	G7G6-B♂	0	2	0		
Total			3	10	15		

G values represent partitions of the total *G* (Table 1) into contributions of individual crosses. Replicated *G*-tests were not possible on cross types that produced very few males.

which hermaphroditism appeared to be dominant to maleness or perhaps to involve multiple loci, a cytoplasmic effect, or differences between populations. We found no evidence of such complexity in this study and suggest that perhaps because crosses were performed outdoors in the previous study, they may have been influenced by pollen contamination, which appears to occur easily in this wind-pollinated species.

There does not appear to be one particular model of sex determination common to all androdioecious and nearly androdioecious species, although in all three plants that have been studied (*M. annua*, Pannell 1997b; *Sagittaria lancifolia*, Muenchow 1998; and *D. glomerata*), there appears to be a single autosomal locus at which maleness is dominant. A similar system (from a population-genetic perspective) is found in the nematode *C. elegans*: Sex is determined by the X to autosome ratio, with hermaphrodites having the XX karyotype and males the XO karyotype (Hodgkin 1983; Kuwabara and Kimble 1992). On the other hand, maleness is recessive in the two androdioecious freshwater shrimp *E. texana* (Sassaman and Weeks 1993) and *Triops newberryi* (Sassaman 1991).

Although it is now clear that androdioecy can exist,

the long-term stability of androdioecy has been questioned due to the strict requirements for the maintenance of males and the low frequencies of males in most androdioecious species (Liston *et al.* 1990; Fritsch and Rieseberg 1992). Thus, androdioecy may persist for long periods only under the most optimal conditions. In a metapopulation simulation, Pannell (1997a) showed that under low rates of extinction and recolonization, androdioecy is more stable if maleness is recessive, whereas the reverse is true under high rates of extinction and recolonization. This is apparently because the male-determining allele will be maintained at a higher frequency if it is recessive rather than dominant. Thus males are less likely to be lost from the metapopulation, at least if populations are long lived. However, if populations are continuously going extinct and being recolonized by hermaphrodites, maleness is more likely to be expressed if it is dominant, and therefore the male-determining allele is less likely to be lost through drift.

According to the results of Pannell (1997a), we might expect to see correlations between metapopulation dynamics and dominance relationships at the sex-determining locus in androdioecious species. Both *M. annua* (Pannell 1997a) and *D. glomerata* (Liston *et al.* 1990) appear to undergo high rates of extinction and recolonization, consistent with the dominant maleness in these species. The clam shrimp, *E. texana*, with recessive maleness, is widespread and abundant, with a persistent bank of encysted embryos in the soil (Sassaman 1989), and thus is likely to have stable, persistent populations, as would be predicted (Pannell 1997a). The widespread *C. elegans*, in which maleness is "dominant," apparently contradicts the prediction. However, males are extremely rare in nature (frequency = 0.001) and are often sterile (Maupas 1900; Honda 1925), apparently being maintained only by recurrent mutations (nondisjunction of X chromosomes, producing gametes with no X; Hodgkin and Doniach 1997). Thus the species cannot be considered functionally androdioecious in nature and supports the theoretical predictions. Although it is premature to make definitive conclusions, on the basis of such a small number of species, there appears to be a relationship between sex-determining mechanisms and metapopulation dynamics as predicted by theory (Pannell 1997a). This relationship may be due to lineage selection; males are more likely to be lost from androdioecious species with suboptimal sex-determining mechanisms.

On the other hand, even if lineage selection creates patterns of sex determination that are consistent with metapopulation dynamics, the sex-determining mechanism of each species is most likely determined by that of its ancestors. When comparisons have been made, it appears that dominance relationships between males and the female-fertile sex in dioecious species are preserved in the presumably derived androdioecious species (Caenorhabditis, Kuwabara and Kimble 1992; Eulimnadia, Sassaman 1995; Mercurialis, Pannell 1997b; and now Datisca).

Females *vs.* hermaphrodites: An important issue concerning the evolution of androdioecy from dioecy is the nature of the mutation allowing hermaphrodites to arise. The mutation could either restore female fertility in males or restore male fertility in females and could be at the primary sex-determining locus that makes the initial developmental switch between males and the female-fertile sex or may be independent, at some downstream regulatory site or at a newly recruited gene. On the basis of observed dominance relationships, we propose (below) that the recessive mutation to hermaphroditism restored male fertility in females, rather than restoring female fertility in males, and that the mutation is probably not within the primary sex-determining locus.

Melandrium album (= *Silene latifolia*) is the best-studied dioecious plant in which the genetic basis of hermaphroditic mutants has been examined. Sex is determined by an active Y, which contains nonrecombining genes that promote androecium formation (male fertility) and suppress gynoecium formation (female sterility; Westergaard 1946, 1958; Lardon *et al.* 1999), such that maleness is dominant to femaleness. Loss or disruption of gynoecium suppressors on the Y creates XY hermaphrodites, in which the male fertility of hermaphrodites is generally dominant to the male sterility of females, just as the male fertility of males is dominant (Westergaard 1946, 1958; Lardon *et al.* 1999). Because in Datisca, the male fertility of males is dominant, whereas the male fertility of hermaphrodites is recessive, the loss of gynoecium suppression is not likely to be the mechanism by which hermaphrodites were generated. Rather, hermaphrodites must have been generated by the restoration of male fertility in females. Thus, we can consider *D. glomerata* hermaphrodites to essentially be females that produce pollen.

Hermaphrodites in the morphologically androdioecious species, *C. elegans*, apparently arose through a mutation independent of the primary sex-determining mechanism, which restored male fertility to females. Because Caenorhabditis males have the XO karyotype, while females and hermaphrodites have an XX karyotype, both males and females (in dioecious species) carry all genes necessary for the expression of either sex. Differences between sexes are due to alternate regulatory cascades induced by the X to autosome ratio. The gene differentiating *C. elegans* hermaphrodites from females in the dioecious *C. remanei* (possibly the regulatory gene *fog-2*; Haag *et al.* 2000) appears to be independent of the primary sex-determining mechanism (X to autosome ratio; Skipper *et al.* 1999) and functions by temporarily activating male gamete-production genes in the hermaphrodite ovotestes (Kuwabara and Kimble 1992; Haag and Kimble 2000). Because the mutation allowing male fertility in females was independent of the primary sex-determining mechanism, the primary sex-determin-

ing mechanism has been conserved in the dioecious and androdioecious species, as we have proposed for Datisca. Further, because Datisca lacks sex chromosomes, there are not likely to be many genes at the primary sex-determining locus, while there are likely to be many unlinked, independent loci not involved in the initial switch between males and females at which mutations could restore male fertility. Thus, hermaphrodites in Datisca may have arisen through a route similar to that seen in Caenorhabditis.

Invasion of hermaphrodites: For androdioecy to evolve from dioecy, hermaphrodite mutations must not only arise, but must be able to invade a dioecious population under selection. Haldane (1927) showed that a new recessive mutation is much less likely to invade a large randomly mating population under selection than is a new dominant mutation, because the phenotype of the recessive mutation will seldom be expressed. Thus, we were surprised by the finding that hermaphroditism is recessive in Datisca. However, the barrier to invasion by recessive mutations can be reduced by inbreeding, which increases the level of homozygosity (Bodmer and Parsons 1960). Thus the small populations often seen in Datisca (Liston *et al.* 1990) and the self-compatibility of hermaphrodites (Fritsch and Rieseberg 1992) may have played an essential role in the invasion of hermaphrodites and the subsequent evolution of androdioecy.

Conversely, we must also consider that if some environmental change occurred so that previously deleterious hermaphrodite alleles became advantageous, alleles maintained at low frequency by mutation-selection balance could invade the population. The introduction of pollen limitation, for instance, could suddenly increase the fitness of previously deleterious hermaphrodite alleles and decrease the fitness of females (Maurice and Fleming 1995). If selection acts on standing variation, the probability of invasion is theoretically independent of dominance for X-linked traits (Orr and Betancourt 2001). For autosomal traits, the probability of invasion is actually higher for completely recessive alleles than for dominant alleles (Orr and Betancourt 2001). This is because deleterious recessive alleles will be maintained through mutation-selection balance at a higher frequency than deleterious dominant alleles. Thus, if androdioecy arose due to a sudden environmental change, we should not be surprised that the newly derived character is recessive.

Whether or not the evolution of androdioecy was precipitated by sudden pollen limitation and a resulting increase in the fitness of existing hermaphrodite alleles (Maurice and Fleming 1995) or by the fortuitous origin of a new and exceptionally fertile hermaphrodite mutation (Charnov 1982; Maurice and Fleming 1995) is unknown. However, investigations into the reproductive output of males, females, and hermaphrodites and forces that could create pollen limitation in these species should give us some insight into the forces allowing the recessive hermaphrodite mutation to invade.

Biased sex ratios: In the dioecious species, *D. cannabina*, all crosses with males from the F collection produced highly female-biased sex ratios (94% female) over two generations, whereas crosses with males from the G collection produced sex ratios that were only slightly, but significantly female biased (54% female). Because all males from the F collection produced the same biased sex ratios, we do not believe that this result contradicts our conclusion of a single sex-determining locus. Rather, it is more likely that some form of sex-ratio factor is involved, such as pollen tube competition (Lloyd 1974), meiotic drive (Carvalho and Klaczko 1994), or maternally inherited elements (Hurst 1993). Elements that enhance female production are generally located on the female-determining sex chromosome (*e.g.*, X chromosome) or are maternally inherited, therefore increasing their own transmission, often at the cost of overall fitness (Werren and Beukeboom 1998; Taylor *et al.* 1999). Due to the reduced transmission of other loci, autosomal or Y-linked suppressors of segregation distortion that partially restore the sex ratio often arise (Taylor 1994b; Carvalho *et al.* 1998).

The difference in sex ratios produced by F and G males can be explained either by variation in the segregation distorter or by variation in a suppressor of distortion. F males may carry an X-linked segregation distorter that G males lack. However, the alternative hypothesis, in which both populations carry a distorter (X-linked or cytoplasmic) and only the G population carries a suppressor, is more likely because the G population also shows a slight excess of females. Additional crosses designed to differentiate between these hypotheses are under way.

Regardless of the mechanism causing biased sex ratios, the finding of heritable female-biased sex ratios in *D. cannabina* proposes an intriguing model for the evolution of androdioecy in this genus. Could genetically induced female-biased sex ratios in ancestral populations have permitted the evolution of androdioecy from dioecy?

Summary: The crossing results and sex-linked AFLP markers lead to the following conclusions: (1) Sex in both dioecious and androdioecious Datisca species appears to be determined by a single, nuclear locus, at which the male-determining allele is dominant; (2) the loci controlling sex determination in both species may be homologous; (3) hermaphroditism is recessive to femaleness and thus must have arisen as a recessive mutation restoring male fertility in females; and (4) some heritable factor, possibly meiotic drive, causes extremely female-biased sex ratios in one collection of the dioecious species.

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