

## THE EVOLUTION AND MAINTENANCE OF MONOECY AND DIOECY IN *SAGITTARIA LATIFOLIA* (ALISMATACEAE)

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**Abstract.**—Plant species rarely exhibit both monoecious and dioecious sexual systems. This limits opportunities to investigate the consequences of combined versus separate sex function on mating patterns and genetic variation and the analysis of factors responsible for the evolution and maintenance of the two sexual systems. Populations of the North American clonal aquatic *Sagittaria latifolia* are usually either monoecious or dioecious and often grow in close geographic proximity. We investigated mating patterns, genetic structure, and relationships between the two sexual systems using allozyme variation in populations from southern Ontario, Canada. As predicted, selfing rates in monoecious populations ( $n = 6$ , mean = 0.41) were significantly higher than in dioecious populations ( $n = 6$ , mean = 0.11). Moreover, marker-based estimates of inbreeding depression ( $\delta$ ) indicated strong selection against inbred offspring in both monoecious (mean  $\delta = 0.83$ ) and dioecious (mean  $\delta = 0.84$ ) populations. However, the difference in selfing rate between the sexual systems was not reflected in contrasting levels of genetic variation. Our surveys of 12 loci in 15 monoecious and 11 dioecious populations revealed no significant differences in the proportion of polymorphic loci ( $P$ ), number of alleles per locus ( $A$ ), or observed and expected heterozygosity ( $H_o$  and  $H_e$ , respectively). Strong inbreeding depression favoring survival of outcrossed offspring may act to maintain similar levels of diversity between monoecious and dioecious populations. Despite geographical overlap between the two sexual systems in southern Ontario, a dendrogram of genetic relationships indicated two distinct clusters of populations largely corresponding to monoecious and dioecious populations. Reproductive isolation between monoecious and dioecious populations appears to be governed, in part, by observed differences in habitat and life history. We suggest that selfing and inbreeding depression in monoecious populations are important in the transition from monoecy to dioecy and that the maintenance of distinct sexual systems in *S. latifolia* is governed by interactions between ecology, life history, and mating.

**Key words.**—Dioecy, gender dimorphism, genetic variation, gynodioecy, monoecy, outcrossing rate, *Sagittaria latifolia*.

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Flowering plants possess diverse strategies that segregate female and male sex function, thus limiting the mating costs associated with self-pollination (Darwin 1877; Lloyd and Webb 1986; Webb and Lloyd 1986; Harder et al. 2000). The production of unisexual flowers in monoecious and dioecious species is perhaps the most obvious means by which self-pollination can be reduced. In dioecious populations, unisexual individuals produce only one type of gamete, thus rendering selfing impossible. However, in monoecious populations, mating patterns are governed by the number of simultaneously presented female and male flowers and, particularly in self-compatible species, rates of self-pollination should vary. Although a large body of data on mating patterns in flowering plants now exists (reviewed in Schemske and Lande 1985; Barrett and Eckert 1990; Barrett et al. 1996), few studies have explicitly contrasted related species with combined versus separate sexes to examine whether differences in the segregation of sex function are associated with contrasting selfing rates. Such differences could have important genetic and evolutionary consequences because mating patterns govern the amount and distribution of genetic variation within and among populations (Hamrick and Godt 1989; Charlesworth and Charlesworth 1995), as well as their response to natural selection (Charlesworth 1992).

In addition to affecting levels of genetic variation, selfing can also influence the evolution of plant sexual systems, especially the evolution of separate sexes from combined sexes (B. Charlesworth and D. Charlesworth 1978; D. Charlesworth and B. Charlesworth 1978; Lloyd 1982; Charlesworth 1999).

For example, models for the evolution of dioecy from monoecy predict that selfing combined with inbreeding depression in cosexual populations favors individuals with reduced allocation to male sex function, and, thus, reduced selfing rates (B. Charlesworth and D. Charlesworth 1978). Individuals with reduced male sex function may arise as a result of complete or partial male-sterility mutations, resulting in the evolution of females or female-biased forms, respectively. These forms are predicted to spread in populations if hermaphrodites self at a high frequency and their offspring suffer large reductions in fitness as a result of inbreeding depression (B. Charlesworth and D. Charlesworth 1978; D. Charlesworth and B. Charlesworth 1978). Significant selfing and inbreeding depression in monoecious populations can therefore provide evidence that inbreeding avoidance has been involved in evolutionary transitions from monoecy to dioecy in lineages that contain both sexual systems.

It is unusual for plant species to maintain both monoecious and dioecious populations (but see Lloyd 1975a; Costich and Meagher 1992; Pannell 1997), although the two sexual systems are commonly associated at the family level (Renner and Ricklefs 1995). The rarity of intraspecific variation restricts opportunities to compare the mating systems and genetic variation of monoecious and dioecious populations and to determine factors responsible for the evolution and maintenance of the two sexual systems without the confounding effects of phylogenetic divergence. The North American clonal aquatic *Sagittaria latifolia* (Alismataceae) is unusual in displaying considerable variation in sexual systems, in-

cluding both dioecious and monoecious populations (Smith 1894; Wooten 1971; Sarkissian et al. 2001). This species therefore provides a valuable opportunity to examine the genetic and evolutionary consequences of combined versus separate sexes.

Several features of *S. latifolia* would be expected to influence mating patterns in monoecious populations. Plants are self-compatible, clonal, and visited by nonspecialist insects (Muenchow and Delesalle 1994). Together, these characteristics should provide ample opportunity for geitonogamous selfing in monoecious populations, especially in clones with large floral displays composed of inflorescences in female and male sexual phases. If monoecious populations experience significant selfing this should result in reduced levels of genetic variation (i.e., reduced allelic richness, proportion polymorphic loci, and heterozygosity) compared to predominantly outcrossing dioecious populations (Wright 1969; Jain 1976; Hamrick and Godt 1989). Moreover, significant levels of selfing and inbreeding depression in monoecious populations may also indicate that inbreeding avoidance has been involved in the transition from monoecy to dioecy in this species.

In southern Ontario, where the present study was conducted, monoecious and dioecious populations of *S. latifolia* occur in close geographic proximity but often occupy different types of aquatic habitats (Sarkissian et al. 2001). Monoecious populations grow in a variety of wetland habitats, including shorelines, creeks, roadside ditches, and ponds associated with agriculture. These habitats are characterized by frequent disturbance and are often ephemeral. In contrast, dioecious populations are primarily restricted to large, permanent wetlands, such as those associated with the Great Lakes and large river systems flowing into them. Where monoecious and dioecious populations occur in close proximity, mixed populations composed of both unisexual and monoecious individuals may exist (Sarkissian et al. 2001). This observation raises the question of how much gene flow occurs between monoecious and dioecious populations and what the genetic relationships between the two sexual systems are in regions of sympatry.

Recurrent gene flow between monoecious and dioecious populations of *S. latifolia* could have important implications for the evolution and maintenance of the two sexual systems. Two potential scenarios could be hypothesized. If gene flow is frequent, comparisons of the genetic distance among populations might be expected to reflect the geographical relationships of populations—and not clustering based on sexual system. However, if gene flow is restricted and the two sexual systems are largely reproductively isolated from one another, we might predict that comparisons of the genetic similarity of populations would reveal groupings based on monoecy versus dioecy, rather than geographical proximity. If the second scenario is correct it would raise the important question as to what mechanisms maintain the integrity of the two sexual systems in an area of geographical overlap.

Here we investigate the consequences of combined versus separate sex function on mating patterns and genetic variation in monoecious and dioecious population of *S. latifolia*. We also use data on the genetic relationships of populations to evaluate the factors responsible for the evolutionary main-

tenance of the two sexual systems. We begin by estimating mating patterns in monoecious and dioecious populations and then examine the magnitude of inbreeding depression using a marker-based approach. To assess whether differences in mating patterns are associated with reduced genetic variation, we compare the distribution of genetic variation within and among 28 populations of *S. latifolia* by measuring allelic richness at allozyme loci, proportion polymorphic loci, and heterozygosity. Finally, we describe the pattern of genetic relationships within and among monoecious and dioecious populations using a dendrogram of genetic distance based on allozyme variation. This data is used to assess alternative hypotheses concerning the extent of reproductive isolation between the two sexual systems.

## MATERIALS AND METHODS

### *Study Species*

*Sagittaria latifolia* is a perennial emergent aquatic common to a variety of wetland habitats (Sarkissian et al. 2001). Both monoecious and dioecious populations exist throughout the species' range (Wooten 1971). In southern Ontario, plants flower between July and mid-September in monoecious and dioecious populations. Flowering individuals produce racemes with three flowers at each node. Inflorescences on monoecious plants produce female flowers at basal nodes and male flowers at distal nodes. Because the single-day flowers open from the bottom to the top of the inflorescence, inflorescences in monoecious populations are protogynous. There is considerable gender variation among populations of *S. latifolia* (Sarkissian et al. 2001). In monoecious populations, this is partly the result of size-dependent gender expression (Sarkissian et al. 2001). In dioecious populations, sex expression remains constant over years and under different growth conditions, however, gender variation can result from low levels of sex inconstancy among male individuals (M. E. Dorken and S. C. H. Barrett, unpubl. data). A variety of insect pollinators visit the flowers of *S. latifolia*, including flies, bees, wasps, and butterflies (Muenchow and Delesalle 1994; M. E. Dorken, pers. obs.).

### *Estimates of Mating Patterns*

To estimate the proportion of selfed versus outcrossed seed among families, we collected seed families from a total of 12 populations (six dioecious and six monoecious) of *S. latifolia* in September and October 1998 (see Fig. 1 for localities). To avoid upward biases of the estimated selfing rate in dioecious populations, we did not sample fruits from rare inconstant males that occurred in several of the dioecious populations. In all populations we collected between 16 and 46 (mean = 26) seed families, each of which were separated by at least 2 m to limit repeated sampling of genets. For each seed family, electrophoretic genotypes were assayed from between 10 and 20 (mean = 13) seeds.

Variation at isozyme loci was resolved using horizontal starch gel electrophoresis. Seeds were crushed in a small volume of 0.1 M Tris-HCl buffer (Soltis et al. 1983). The resulting homogenate was absorbed on chromatography-paper wicks (3 mm, Whatman, Maidstone, U.K.), and placed

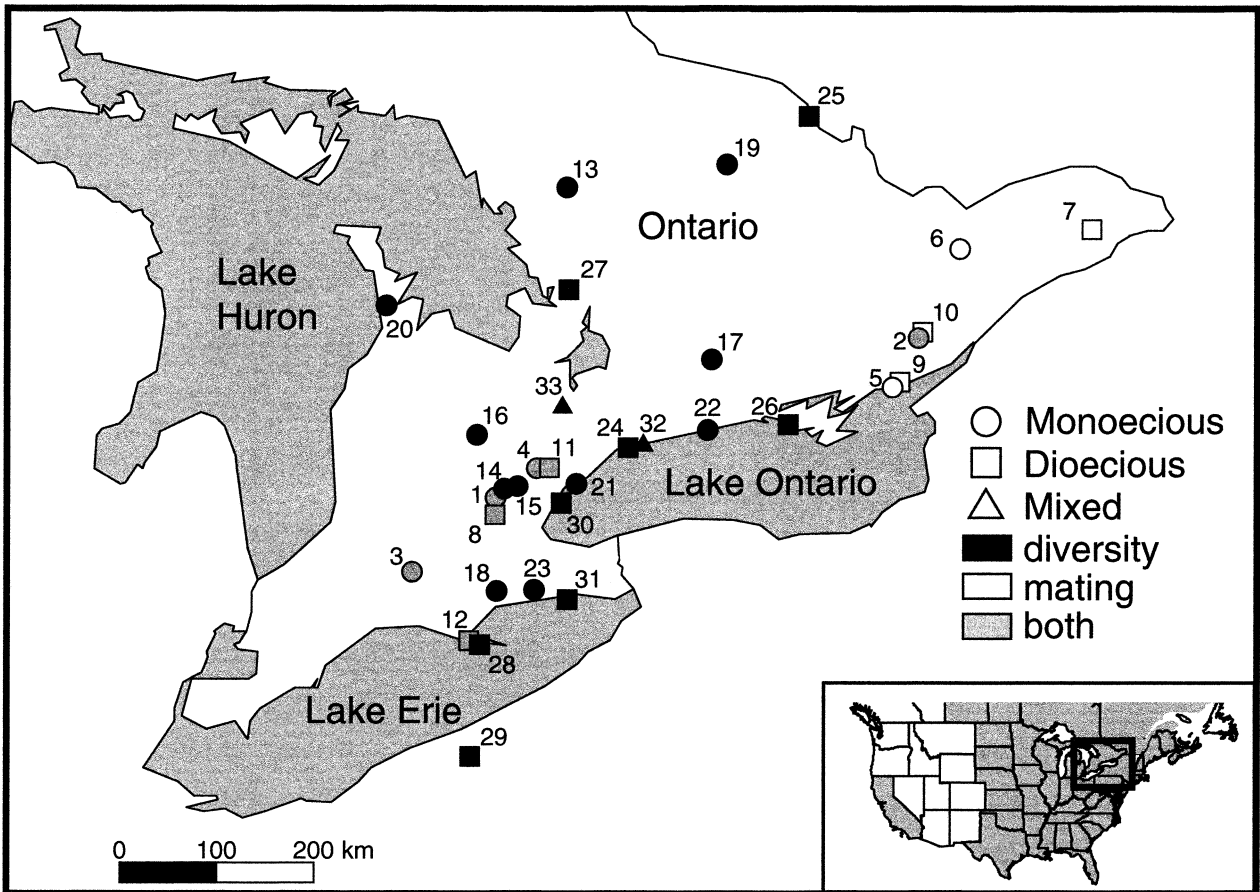


FIG. 1. Localities of *Sagittaria latifolia* populations sampled for genetic variation (black symbols) and outcrossing rates (open symbols). Seven populations were sampled for both genetic variation and outcrossing rates (shaded symbols). Shaded areas in the inset indicate states and provinces in which *S. latifolia* occurs (modified from Haynes and Hellquist 2000). Population numbers correspond with those in Table 1, Figure 2, and Figure 3.

directly onto 11–12% starch gels. From an initial screen of 18 loci, we resolved four variable loci using two electrode buffer systems: a histidine-citrate buffer (pH 6.5) was used to resolve isocitrate dehydrogenase (*Idh*) and phosphoglucumutase (*Pgm*); a lithium-borate buffer (pH 8.3) was used to resolve glutamate oxaloacetate transaminase (*Got*) and phosphoglucose isomerase (*Pgi*). Gels were stained for enzyme activity following recipes in Wendel and Weeden (1991). Three alleles were detected at the *Idh* and *Pgm* loci, two alleles at the *Got* and *Pgi* loci. Genotypes were inferred based on segregation patterns characteristic of either dimeric or monomeric codominant enzymes.

For each population, we estimated the outcrossing rate ( $t$ ), the complement of the selfing rate ( $s$ ; i.e.,  $t = 1 - s$ ) and the parental inbreeding coefficient ( $F$ ) using the computer program MLTR (vers. 0.9, Ritland 1986). This program uses maximum-likelihood procedures to infer the genotypes of the maternal parents, allele frequencies in the pollen pool, and the proportion of progeny that are the result of outcrossing. Parental inbreeding coefficients are calculated based on inferred maternal genotypes. Standard errors of the outcrossing rate and inbreeding coefficient estimates were derived from the standard deviation of 1000 bootstrap values, using the seed family as the unit of resampling. Expectation-maximi-

zation iteration was used to find maximum-likelihood estimates of the outcrossing rate. For two populations (M-6 and D-10), only one variable locus was detected, and outcrossing rates were estimated using the single locus. For the remaining populations, between two and four loci (mean = 2.4) were used to estimate the outcrossing rate. Inbreeding depression [ $\delta = 1 - (\text{fitness of selfed progeny}/\text{fitness of outcrossed progeny})$ ] for survival from seed to reproductive maturity was calculated from the estimates of the outcrossing rate ( $t$ ) and parental inbreeding coefficients ( $F$ ) using Ritland's (1990) equilibrium estimator:

$$\delta = 1 - \left[ \frac{2tF}{(1-t)(1-F)} \right]. \quad (1)$$

To determine whether estimates of the parental inbreeding coefficient and inbreeding depression were significantly greater than 0.0 and 0.5, we examined the distribution of 1000 bootstrap values following methods outlined in Eckert and Barrett (1994a). We tested whether values for the parental inbreeding coefficient were significantly greater than 0.0 and whether values for inbreeding depression were significantly greater than 0.0 and 0.5 by examining the distribution of bootstrap values. Using this method, parameter estimates for each population were considered to be significantly higher



TABLE 1. Mean ( $\pm$ SE) parental inbreeding coefficient ( $F$ ) and inbreeding depression estimates ( $\delta$ ) for 12 populations of *Sagittaria latifolia*. For each population, the number of maternal families ( $N$ ) and the number of variable loci detected ( $n$  loci) are presented. Averages for monoecious and dioecious populations are also presented. The average  $\delta$  for dioecious populations was calculated excluding the estimate for D-10. Sexual system averages followed by different letter superscripts are significantly different from one another based on the distribution of bootstrap values (see Materials and Methods).

Population	$N$	$n$ loci	$F$	$\delta$
<b>Monoecious</b>				
M-1	28	2	0.10 (0.09)	0.87 (0.22)
M-2	18	2	0.19 (0.14)	0.69 (0.60)
M-3	21	3	0.14 (0.10)	0.63 (0.36)
M-4	26	3	0.05 (0.08)	0.80 (0.26)*
M-5	45	3	0.000 (0.00)	1
M-6	19	1	0.000 (0.00)	1
<b>Dioecious</b>				
D-7	18	3	0.03 (0.03)	0.77 (1.86)
D-8	18	2	0.000 (0.00)	1
D-9	16	2	0.13 (0.11)	-1.19 (3.17)
D-10	38	1	0.001 (0.00)	0.98 (0.21)**
D-11	27	3	0.002 (0.00)	0.94 (0.01)**
D-12	39	4	0.01 (0.01)	0.52 (6.91)
<b>Averages for each sexual system</b>				
Monoecious	26.2	2.3 (0.33)	0.08 <sup>a</sup> (0.02)*	0.83 <sup>a</sup> (0.13)**
Dioecious	28.0	2.6 (0.43)	0.03 <sup>b</sup> (0.02)	0.84 <sup>a</sup> (1.38)

\* Estimates that are significantly greater than 0.

\*\* Estimates that are significantly greater than 0.0 and 0.5.

than 0.0 or 0.5 if  $100[1 - (\alpha_{PC}/2)]$  percent of the bootstrap values were all greater than 0.0 or 0.5 (where  $\alpha_{PC}$  represents the Type I error rate per contrast). For three populations (D-8, M-5, and M-6) no variation in the parental inbreeding coefficient was detected, resulting in uniform estimates of  $\delta = 1$ . For these populations, statistical departures from 0.0 and 0.5 were not performed because of nonvariable bootstrap estimates. Overall differences in the outcrossing rate between monoecious and dioecious populations were tested in a similar manner. Monoecious and dioecious populations were considered to have significantly different outcrossing rates if, as above,  $100[1 - (\alpha_{PC}/2)]$  percent of the difference in average bootstrap estimates between the six monoecious and five of the six dioecious populations for each parameter were either all greater than zero or all less than zero (the pooled estimate of inbreeding depression for dioecious populations was calculated excluding the estimate from population D-9, which was considered unreliable because of the small sample size and large standard error; see Table 1).

### Genetic Variation

To quantify levels of genetic variation among populations of *S. latifolia*, we sampled ramets from 28 populations in southern Ontario plus one dioecious population from Pennsylvania in 1998 and 1999 (see Fig. 1. for localities). All populations except two (I-32 and I-33) were easily classified as either monoecious or dioecious. These two populations contained a substantial number of both unisexual and hermaphroditic individuals and were classified as mixed populations following Sarkissian et al. (2001). Leaf material from between five and 36 individuals per population (mean = 16.8)

was sampled from 15 monoecious populations, 11 dioecious populations, and two mixed populations. There was no difference in the mean number of individuals sampled between monoecious (mean = 16.7) and dioecious (mean = 16.1) populations (one-way ANOVA,  $F_{1,24} = 0.04$ ,  $P > 0.8$ ). In addition, leaf material from two populations of the closely related monoecious *Sagittaria cuneata* (Bogin 1955) was sampled to allow a comparison of genetic distance (see below) between sexual systems versus between species. Leaves from each population were ground in five or six drops of 0.1M Tris-HCl extraction buffer (Soltis et al. 1983), and the extract absorbed onto 1.5- and 3-mm chromatography paper wicks and stored at  $-80^{\circ}\text{C}$  until electrophoresis could be performed.

We detected more loci using fresh leaf material than from the seeds assayed for the estimation of mating patterns. From an initial screen of 18 enzyme encoding loci, we resolved 12 variable loci. We used a lithium borate buffer system (pH 8.3) to resolve alcohol dehydrogenase (*Adh*), diaphorase (*Dia*), glutamate dehydrogenase (*Gdh*), *Got*, peroxidase (*Per*), superoxide dismutase (*Sod*), and triose-phosphate isomerase (*Tpi*) and a histidine-citrate buffer system (pH 6.5) to resolve aconitate hydratase (*Aco*), *Idh*, *Pgi*, *Pgm*, and 6-phosphogluconate dehydrogenase (*6Pgd*). Individuals were randomized before electrophoresis and coded to ensure that scoring of isozyme genotypes was blind with respect to sexual system.

Genetic variation was estimated as the percentage of polymorphic loci ( $P$ ), the number of alleles per locus ( $A$ ), and the observed and expected heterozygosity ( $H_o$  and  $H_e$ , respectively). These measures were computed using BIOSYS (ver. 1.7, Swofford and Selander 1981). We investigated differences between monoecious and dioecious populations for each of the measures of genetic variation using one-way analysis of covariance of population means. The average sample size per locus was used as the covariate for each analysis. Because levels of genetic variation may vary with population size, we used the mean of two independent estimates of the number of ramets per population as a measure of population size (data not shown). These estimates were log-transformed and used to analyze the correlation between population size and each measure of genetic variation. We calculated  $f$ ,  $\theta$ , and  $F$ , parameters analogous to Wright's (1922) inbreeding coefficients  $F_{IS}$ ,  $F_{ST}$ , and  $F_{IT}$ , respectively (Weir and Cockerham 1984), using FSTAT (ver. 3.0, Goudet 1995) to quantify the distribution of genetic variation within and among populations. Unbiased jackknifed estimates for these estimates of the inbreeding coefficients ( $\hat{F}_{IS}$ ,  $\hat{F}_{ST}$ ,  $\hat{F}_{IT}$ ) and their associated standard errors were based on 1000 permutations of the data within loci ( $\hat{F}_{IS}$ ) and across genotypes within loci ( $\hat{F}_{ST}$  and  $\hat{F}_{IT}$ ).

### Genetic Relationships

To investigate patterns of genetic relationships among the 28 populations of *S. latifolia* and two populations of *S. cuneata*, we calculated genetic distances derived from the allele frequencies measured above (Nei 1978). These genetic distance measures were then analyzed by producing a dendrogram using the unweighted pair-group clustering method

(UPGMA, Sneath and Sokal 1973). Allele frequencies and genetic distances were computed using the BIOSYS program (ver. 1.7, Swofford and Selander 1981). Standard errors for each genetic distance measure were calculated following the methods outlined in Ritland (1989).

We tested for isolation by distance among populations within monoecious and dioecious sexual systems, as well as between the two sexual systems by determining the correlation between geographic distance and genetic distance using Mantel's tests (Smouse et al. 1986). To avoid biasing the estimate of the correlation between geographic and genetic distance between dioecious and monoecious populations, only pairs of monoecious and dioecious populations were considered. Tests of significance for each correlation were based on 1000 permutations of the distance matrices. Correlations were considered significant if  $\alpha > [(n_T + 1)/(N + 1)]$ , where  $\alpha$  is the Type I error rate,  $n_T$  is the number of randomized  $r$ -values equal to or greater than the observed  $r$ , and  $N$  is the number of replicated randomizations (Sokal and Rohlf 1995). Because different numbers of monoecious and dioecious populations were used in the analysis of isolation by distance between monoecious and dioecious populations, our distance matrices were not square and symmetrical. Therefore standard Mantel's tests could not be used. Instead, each permutation was based on randomizing the data in both rows and columns. To determine whether this modification of the Mantel's test introduced a bias, we compared our results to those obtained using standard Mantel's tests as calculated by GENEPOP (ver. 3, Raymond and Rousset 1995). For the correlation between genetic and geographic distance for pairs of monoecious and dioecious populations, we compared our results to those obtained from GENEPOP in which the distance matrices were made square by the removal of randomly chosen populations. For the correlation between genetic and geographic distance among monoecious and among dioecious populations, we compared our results to those obtained from GENEPOP directly. Our results did not differ from those calculated using GENEPOP, and the data presented below are based on random permutations of both rows and columns.

## RESULTS

### *Mating Patterns*

Among all populations of *S. latifolia*, the outcrossing rate ranged from 0.37 to 0.96 (mean =  $0.74 \pm 0.06$  SE; Fig. 2). On average, monoecious populations had significantly lower outcrossing rates (mean =  $0.59 \pm 0.05$  SE) than dioecious populations (mean =  $0.89 \pm 0.03$ ; one-tailed  $P < 0.001$ ). The same result was found if we excluded the two populations (M-6 and D-10) for which only single-locus estimates of the outcrossing rate were available (one-tailed  $P < 0.001$ ). However, there was considerable variation in outcrossing rates among populations within sexual systems, particularly among monoecious populations in which  $t$  ranged from 0.37 to 0.87, (compared to dioecious populations, 0.79 to 0.96).

Parental inbreeding coefficients varied among populations but were generally low among both monoecious and dioecious populations (average  $F = 0.05 \pm 0.02$  SE; Table 1). On average, inbreeding coefficients in monoecious popula-

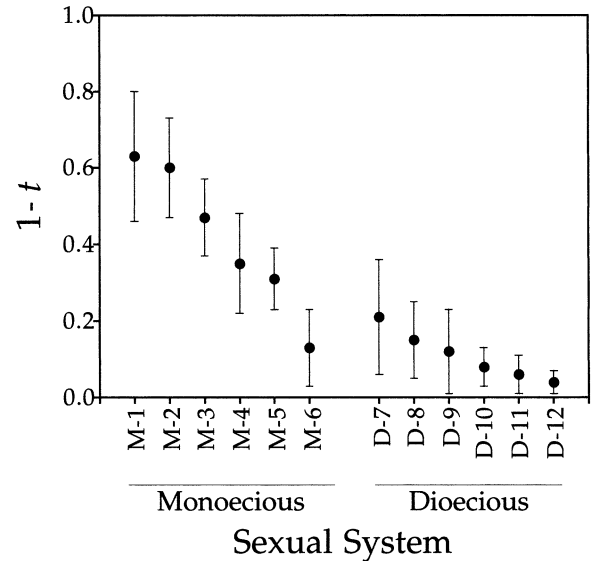


FIG. 2. Distribution of selfing rates ( $1 - t$ ) for six dioecious and six monoecious populations of *Sagittaria latifolia*. The data shown are the maximum-likelihood means ( $\pm$ SE). Standard errors were calculated from the distribution of 1000 bootstraps (Ritland 1986).

tions were significantly greater than in dioecious populations (Table 1). Among monoecious populations, the inbreeding coefficient was significantly greater than zero (Table 1). Among all populations, there was a negative, but nonsignificant correlation between the parental inbreeding coefficient and the outcrossing rate (Spearman  $r_s = -0.55$ ,  $P = 0.10$ ).

### *Inbreeding Depression*

Using a marker-based approach, we found evidence for substantial inbreeding depression in both monoecious (mean  $\delta = 0.83$ ) and dioecious populations (mean  $\delta = 0.84$ ; this pooled value was calculated excluding the estimate from population D-9; Table 1). The average estimates of  $\delta$  for monoecious and dioecious populations were not significantly different ( $P = 0.31$ ). For monoecious populations, the average estimate of  $\delta$  was significantly greater than 0.5 ( $P = 0.03$ ). In dioecious populations, the average estimate of  $\delta$  was only marginally different from 0.0 ( $P = 0.06$ ). However, this result was due to the relatively large standard error associated with dioecious population D-12. Removal from the analysis of this population (in addition to D-9) resulted in an estimate of  $\delta$  significantly greater than 0.5 among the four remaining dioecious populations ( $P = 0.05$ ).

### *Genetic Variation*

There was no significant difference in all four measures of genetic variation between monoecious and dioecious populations, although for all four there was a trend for higher values of genetic variation in dioecious populations ( $P$ :  $F_{1,23} = 2.3$ ,  $P = 0.14$ ;  $A$ :  $F_{1,23} = 0.8$ ,  $P = 0.37$ ;  $H_o$ :  $F_{1,23} = 2.6$ ,  $P = 0.12$ ;  $H_e$ :  $F_{1,23} = 0.7$ ,  $P = 0.40$ ; Table 2). Among all populations, two of the four measures of genetic variation,  $P$  and  $A$ , were correlated with estimated population size ( $P$ :  $r = 0.63$ ,  $P < 0.001$ ;  $A$ :  $r = 0.46$ ,  $P < 0.05$ ;  $H_o$ :  $r = 0.16$ ,

TABLE 2. Comparison of genetic variation in *Sagittaria latifolia* populations with different sexual systems. The values shown are the mean ( $\pm$ SE) number of individuals sampled per population ( $\bar{n}$ ), the percentage of loci polymorphic averaged across populations ( $P$ ), the mean number of alleles per locus ( $A$ ), the observed heterozygosity ( $H_o$ ), and the expected heterozygosity ( $H_e$ ).

Sexual system	$\bar{n}$	$P$	$A$	$H_o$	$H_e$
Monoecious	16.7 (2.3)	58.9 (4.1)	1.8 (0.1)	0.15 (0.02)	0.19 (0.01)
Dioecious	16.1 (1.8)	65.9 (3.6)	1.9 (0.1)	0.20 (0.02)	0.21 (0.02)
Mixed	21.2 (11.4)	79.2 (4.2)	2.1 (0.3)	0.16 (0.03)	0.23 (0.02)

$P = 0.42$ ;  $H_e$ :  $r = 27$ ,  $P = 0.18$ ). Finally, there was no significant difference between monoecious and dioecious populations in  $\hat{F}_{IS}$  and  $\hat{F}_{ST}$ , however,  $\hat{F}_{IT}$  was significantly higher in monoecious compared to dioecious populations (Table 3).

### Genetic Relationships

A UPGMA cluster analysis revealed three broad groups of populations (Fig. 3): one group that distinguishes *S. cuneata* from *S. latifolia* (mean genetic distance = 0.32, range = 0.25–0.46) and two groups that distinguish, for the most part, monoecious from dioecious populations (mean genetic distance between monoecious and dioecious populations = 0.15, range = 0.03–0.31). Only one monoecious population (M-2) had greater similarity to dioecious populations than to other monoecious populations. One of the mixed populations (I-33) had greater similarity to monoecious populations. The other mixed population (I-32) had greater similarity to dioecious populations (Fig. 3). The average genetic distance among all *S. latifolia* populations was 0.17 (range = 0.01–0.34). Among monoecious populations the mean genetic distance was 0.09 (range = 0.01–0.34), and among dioecious populations, the mean genetic distance was 0.06 (range = 0.01–0.13). There were no fixed differences in allelic composition between monoecious and dioecious populations. In contrast, four alleles characteristic of *S. cuneata* were not found in any population of *S. latifolia*.

There was no correlation between geographic and genetic distance among all pairs of monoecious and dioecious populations ( $r = 0.07$ ,  $P = 0.33$ ). Similarly, there was no correlation between geographic distance and genetic distance among dioecious populations ( $r = 0.02$ ,  $P = 0.44$ ). However, for monoecious populations, there was a significant positive correlation between geographic distance and genetic distance ( $r = 0.34$ ,  $P = 0.001$ ).

### DISCUSSION

Despite considerable interest in the evolution of gender dimorphism in flowering plants, little is known about the evolutionary pathway from monoecy to dioecy. Our study provides the first evidence in support of a role for inbreeding

avoidance in the transition from monoecy to dioecy. We show that monoecious populations of *S. latifolia* can experience substantial levels of selfing and inbreeding depression, a result consistent with the hypothesis that avoidance of inbreeding is involved in the evolution of dioecy in this species. However, in spite of variable selfing rates we found that, on average, monoecious populations contain similar levels of genetic variation compared to dioecious populations. This result may be due to the failure of selfed offspring to survive in monoecious populations, preventing increased levels of homozygosity among monoecious populations. Finally, our comparison of the genetic relationships between monoecious and dioecious populations indicates that in southern Ontario there is limited gene flow between the two sexual systems despite their close geographical proximity. We propose that the maintenance of the two sexual systems results from reproductive isolation through habitat differentiation. Below, we discuss each of these results and their interpretations in detail, as well as their implications for the evolution and maintenance of sexual systems in *S. latifolia*.

### Patterns of Mating

Our study is the first to use genetic markers to compare mating patterns in conspecific monoecious and dioecious plant populations. In general, outcrossing rates might be expected to be lower in cosexual populations of self-compatible plants compared to dioecious populations. However, inferences on mating patterns in monoecious populations are by no means straightforward without detailed information on floral biology. Spatial and temporal segregation of the sexes as a result of dicliny and inflorescence-level dichogamy, respectively, should reduce opportunities for self-pollination in monoecious populations (Harder et al. 2000). However, previous research on *S. latifolia* found extensive overlap in sex expression within monoecious inflorescences (Delesalle and Muenchow 1992), but this work was conducted on monoecious individuals in otherwise gender dimorphic populations. Our own observations of monoecious populations indicate that female flowers on inflorescences open more or less synchronously, usually at least one day before male flowers (M. E. Dorken and S. C. H. Barrett, pers. obs.). Oppor-

TABLE 3. Fixation indices for monoecious and dioecious populations of *Sagittaria latifolia*. The data presented are the unbiased jackknife means (and their 95% confidence limits) calculated from the distribution of 1000 jackknife and 1000 bootstrap estimates, respectively, for 15 monoecious and 11 dioecious populations of *S. latifolia*.

Sexual system	$\hat{F}_{IS}$	$\hat{F}_{ST}$	$\hat{F}_{IT}$
Monoecious	0.29 (0.13–0.45)	0.29 (0.22–0.35)	0.50 (0.39–0.60)
Dioecious	0.11 (0.05–0.17)	0.19 (0.12–0.25)	0.28 (0.20–0.33)



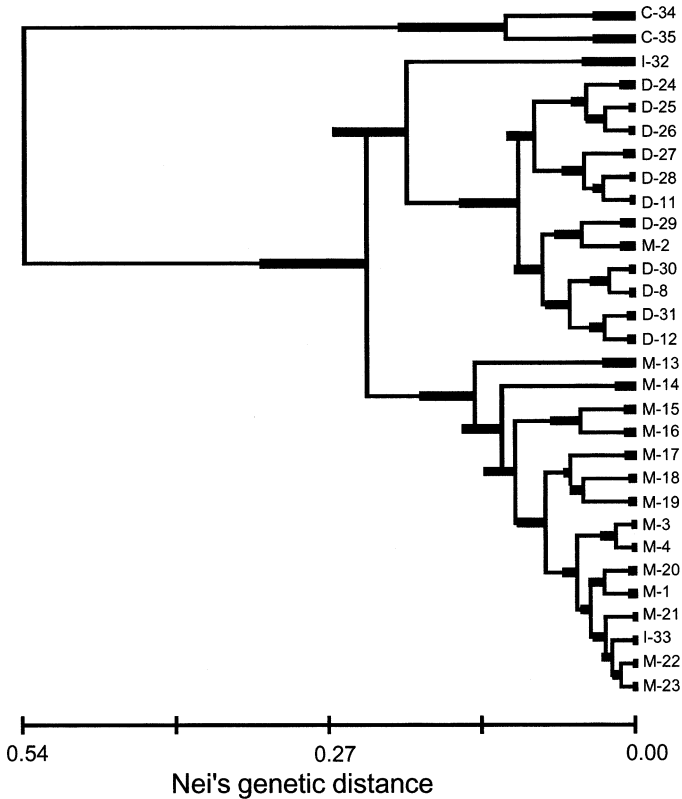


FIG. 3. Genetic relationships among 28 populations of *Sagittaria latifolia* and two populations of *S. cuneata*. The UPGMA dendrogram of Nei's (1978) genetic distance was based on isozyme variation at 12 loci. Thicker bars on dendrogram branches indicate one standard error (Ritland 1989). Population types are identified by a one-letter prefix (C, *S. cuneata*; I, mixed populations; M, monoecious populations; D, dioecious populations of *S. latifolia*).

tunities for intra-inflorescence selfing are therefore probably quite limited, although observation of seed set on isolated inflorescences indicates that some overlap of sex function must occur (S. C. H. Barrett, pers. obs.).

Our analysis revealed wide variation in outcrossing rates associated with the two sexual systems of *S. latifolia*. In monoecious populations, outcrossing rates were significantly lower than dioecious populations and ranged from mixed mating to primarily outcrossing. In two monoecious populations, more than half of the seeds assayed resulted from self-fertilization. Ramets of *S. latifolia* produce one inflorescence at a time and it therefore seems likely that high selfing results primarily from interinflorescence geitonogamy. Individual clones can be composed of several flowering ramets and the promiscuous pollinators of *S. latifolia* forage locally visiting both male- and female-phase inflorescences. Eckert (2000) reported that over 60% of the selfing in the clonal aquatic *Decodon verticillatus* was due to geitonogamous pollen transfer between branches, and a recent study of the clonal sea grass *Zostera marina* demonstrated higher selfing associated with large clone size (Reusch 2001). A plausible explanation for the observed variation among populations of *S. latifolia* in selfing rate is that it is associated with different intensities of interinflorescence geitonogamy. Estimates of

clone size in monoecious populations would be valuable in assessing this hypothesis.

While outcrossing rates in dioecious populations of *S. latifolia* were significantly higher than monoecious populations, nonzero selfing rates were recorded in all populations (Fig. 2). Such apparent selfing rates have been reported elsewhere in females of sexually dimorphic species. Kohn and Biardi (1995) found selfing rates of 0.41 and 0.22 in females from two gynodioecious populations of *Cucurbita foetidissima*. Similarly, Sun and Ganders (1988) reported selfing estimates ranging between 0.00 and 0.25 among females of five gynodioecious species of *Bidens*. Nonzero estimates of the selfing rate among females in gender dimorphic populations may result from a combination of sex inconstancy, biparental inbreeding, and population substructure. Because we avoided sampling sex-inconstant individuals in dioecious populations, there were no opportunities for true selfing to occur in our sample. Thus, some combination of biparental inbreeding and population substructure likely resulted in the nonzero estimates of the selfing rate that we obtained in dioecious populations of *S. latifolia*.

#### Genetic Variation

Our comparison of measures of genetic variation revealed no major differences between the two sexual systems, despite higher selfing rates in monoecious compared to dioecious populations. Although the four indices of genetic variation used in this study ( $P$ ,  $A$ ,  $H_o$  and  $H_e$ ) were always lower in monoecious compared to dioecious populations, the differences were not statistically significant (Table 2). Our results contrast with those from the only other study to compare levels of genetic variation in monoecious versus dioecious populations of the same species. Costich and Meagher (1992) reported substantially less genetic variation among monoecious populations of *Ecballium elaterium* subsp. *elaterium* than dioecious populations of *E. elaterium* subsp. *dioicum*. Costich and Meagher (1992) attributed this difference to the ability of single individuals from the monoecious subspecies to establish new populations and the greater likelihood that individuals from the monoecious subspecies are inbred compared to those from the dioecious subspecies. Indeed, populations of the monoecious subspecies have greater values of the inbreeding coefficient than populations of the dioecious subspecies (Costich and Meagher 1992). However, because they did not measure the selfing rate directly, it is unclear whether higher values of the inbreeding coefficient in the monoecious subspecies result from increased rates of selfing or from their greater susceptibility to founder events and genetic drift in small, recently established populations.

In our study, we have shown significantly higher selfing rates in monoecious compared to dioecious populations of *S. latifolia*; however, this increase was not associated with reduced genetic variation. This result is puzzling because unlike dioecious populations of *S. latifolia*, monoecious populations are often found in ephemeral aquatic habitats such as ditches and farm ponds, suggesting that cosexuality may be important in colonization success. We therefore expected that a combination of frequent founder events and selfing would lead to reduced levels of genetic variation in monoecious popu-

lations compared to dioecious populations. Our inability to detect significantly lower levels of genetic variation in monoecious populations therefore requires explanation.

There are two possible explanations for the maintenance of roughly equivalent levels of genetic variation in monoecious versus dioecious populations of *S. latifolia*. First, genetic variation could be maintained in monoecious populations by substantial gene flow. However, the level of  $\hat{F}_{ST}$  measured in monoecious populations (0.29) indicates that gene flow is likely to be quite restricted among monoecious populations. Indeed this value indicates a fairly high level of differentiation among populations, a pattern not unexpected in a colonizing plant. In addition, comparisons of the genetic relationships among monoecious populations indicate there is a positive correlation between genetic distance and geographic distance (see Results). Such isolation by distance would seem to rule out the possibility that pervasive gene flow among monoecious populations acts to continually restore diversity lost through cycles of colonization.

A second explanation for the similar levels of genetic variation in populations of the two sexual systems of *S. latifolia* concerns the effects of inbreeding depression in monoecious populations. If selfed progeny rarely contribute to the adult mating pool because of low viability, then decreased genetic variation as a result of lower outcrossing rates would not be realized. However, such an effect would require high levels of inbreeding depression. Previous estimates of inbreeding depression from two populations of *S. latifolia* by Delesalle and Muenchow (1992) found that selfed progeny are on average about 75% as fit as outcrossed progeny, probably not high enough to prevent reductions in genetic variation due to inbreeding. However, their study was conducted under glasshouse conditions and involved a restricted number of early stages in the life history (e.g., seed germination and survival). It is possible that under field conditions lifetime estimates of the fitness of selfed and outcrossed offspring would reveal much stronger inbreeding depression (Dudash 1990; Husband and Schemske 1996).

To avoid the inherent problems of experimental measures of inbreeding depression (e.g., difficulties of obtaining lifetime estimates and the environment-dependent nature of inbreeding depression), we investigated this parameter in *S. latifolia* using an indirect, marker-based approach (Ritland 1990; Eckert and Barrett 1994b). Although the amount of variation in estimates of the parental inbreeding coefficient reduces our confidence in individual population estimates of inbreeding depression (Table 1; see also Eckert and Barrett 1994b; Kohn and Biardi 1995; Routley et al. 1999), the consistently high values that we obtained for both monoecious and dioecious populations suggests that values of inbreeding depression are substantial in *S. latifolia*. The average estimate of inbreeding depression in monoecious populations was 0.83, indicating that selfed progeny may be only about 17% as fit as outcrossed progeny. Moreover, estimates of the parental inbreeding coefficient in monoecious populations were not significantly different from zero, further supporting the view that inbred offspring rarely contribute to the adult mating pool. If so, selfing rates would be expected to have minimal effects on levels of genetic variation and this could explain why populations of the two sexual systems have sim-

ilar values for the population genetic parameters we measured. However, if selfed offspring in monoecious populations do indeed suffer strong inbreeding depression, it raises doubts concerning the potential role of cosexuality, and thus selfing and reproductive assurance in colonizing populations of *S. latifolia*. Additional studies of selfing and inbreeding depression in monoecious populations of *S. latifolia* are clearly warranted.

The reliability of our estimates of inbreeding depression is based on satisfying two main assumptions (Ritland 1990). First, the populations studied are assumed to be at inbreeding equilibrium. If the selfing rate is increasing among the populations of *S. latifolia* used in this study, this would have resulted in an upward bias in our estimate of inbreeding depression (Eckert and Barrett 1994b). Although we cannot directly address whether we have violated this assumption, the assumption would be supported by a negative correlation between the outcrossing rate and the parental inbreeding coefficient among populations (Routley et al. 1999). Although we found a negative correlation, it was not statistically significant (see Results). The second main assumption is that the selfing rate and inbreeding coefficient do not vary greatly between years. If we assume that fluctuations in the selfing rate among years are analogous to the fluctuation among populations (Ritland 1990), the large differences in selfing rate between populations suggest that year-to-year variation in the selfing rate, particularly among monoecious populations, may be substantial. However, because violation of this assumption leads to underestimates of the magnitude of inbreeding depression (see Ritland 1990), we may in fact have underestimated this parameter in populations of *S. latifolia*.

#### *Reproductive Isolation between Monoecious and Dioecious Populations*

The results of this study suggest that monoecious and dioecious populations of *S. latifolia* in southern Ontario are largely reproductively isolated from one another. There are two lines of evidence that support this inference. First, genetic distance between pairs of monoecious and dioecious populations is not correlated with their geographic proximity. Second, monoecious and dioecious populations largely represent distinct clusters in the UPGMA analysis (Fig. 3). The average genetic distance between monoecious and dioecious populations of *S. latifolia* is 0.15. This compares with a value of 0.19 for the progenitor-derivative species pair *S. isoetiformis* and *S. teres* (Edwards and Sharitz 2000). For *S. isoetiformis* and *S. teres*, reproductive isolation is governed by nonoverlapping geographic ranges and a lack of cross-compatibility (Edwards and Sharitz 2000). However, the mechanisms governing reproductive isolation in *S. latifolia* appear to be more subtle than those governing isolation between *S. isoetiformis* and *S. teres*.

First, there are no fixed differences in allelic composition between monoecious and dioecious populations of *S. latifolia*. Thus, the genetic distance between monoecious and dioecious populations is based entirely on differences in allele frequencies. This suggests that, although contemporary gene flow between the two sexual systems may be restricted, isolation has not been sufficient to enable fixation of unique



alleles at allozyme loci. Second, crosses that we have made both within and between the two sexual systems indicate that monoecious and dioecious populations of *S. latifolia* are cross-compatible, and the F<sub>1</sub> offspring from these crosses are fully fertile (M. E. Dorken and S. C. H. Barrett, unpubl. data). In addition, our observations to date of plants in mixed populations provide no evidence that plants belonging to the two sexual systems are intersterile and therefore represent different biological species. Finally, whereas monoecious and dioecious populations of *S. latifolia* have overlapping geographic ranges, they grow in different types of aquatic habitats. Monoecious populations are commonly found in ephemeral and disturbed aquatic habitats. In contrast, dioecious populations are largely restricted to permanent wetlands and large river systems (Wooten 1971; Sarkissian et al. 2001; M. E. Dorken and S. C. H. Barrett, unpubl. data). Our common-garden and field-transplant studies have demonstrated that plants from monoecious versus dioecious populations possess different life-history traits that appear to favor reproduction and survival in their respective habitats (M. E. Dorken and S. C. H. Barrett, unpubl. data). Thus, it seems most likely that reproductive isolation between monoecious and dioecious populations may be largely governed by selection for growth and reproduction in the different types of aquatic habitats occupied by each sexual system and not by hybrid inviability or sterility.

The one exception to the overall pattern of genetic differentiation between monoecious and dioecious populations of *S. latifolia* is M-2, a monoecious population that clustered with dioecious populations (Fig. 3). This population grows in a roadside ditch within 1 km of several large, dioecious populations associated with the lakes and waterways of the Rideau Canal. Interestingly, this particular population was included in a recent study of size-dependent gender modification in monoecious and dioecious populations of *S. latifolia* (Sarkissian et al. 2001, population 6). This study demonstrated that plants in this population exhibited an unusual pattern of size-dependent allocation to male flower production that resembled patterns observed in dioecious populations. Male flower number was positively correlated with ramet size rather than remaining independent of size, as was observed in most monoecious populations. The close geographic proximity and genetic similarity to dioecious populations, plus similar allocation patterns, indicate that this monoecious population has almost certainly arisen following the establishment of an inconstant male from a nearby dioecious population. If so, this represents an example of a reversion from dioecy to monoecy. Such reversions, although rare, are not unexpected in gender dimorphic species with sex inconstancy such as *S. latifolia*. Indeed, reversions from gender dimorphism to monomorphism have been reported elsewhere in several other taxa (Lloyd 1975a; Sytsma et al. 1991; Weller et al. 1995), casting doubt on the common assumption that the origin of dioecy represents an irreversible evolutionary pathway (Bull and Charnov 1985).

#### *Implications for the Evolution of Dioecy*

The trait most commonly associated with dioecy at the family level is monoecy (Renner and Ricklefs 1995), sug-

gesting that the transition from monoecy to dioecy is the most common pathway for the evolution of dioecy. Unisexual flowers are already present in monoecious populations, and dioecy may evolve more easily from monoecy than from ancestors with hermaphroditic flowers. Accordingly, the evolution of dioecy from monoecy may not require the spread of mutations of large effect. Instead selection on genes of small effect resulting in changes in floral sex ratios may be largely involved (Charlesworth 1999). However, a recent phylogenetic analysis of the evolution of dioecy in the monocotyledons reported that most sister taxa of dioecious species possess hermaphroditic flowers, implicating the gynodioecy pathway as most important for the evolution of dioecy (Weiblen et al. 2000). Moreover, Sarkissian et al. (2001) recently suggested that this pathway may be involved in the evolution of dioecy from monoecy in *S. latifolia* (and see below).

Regardless of whether dioecy evolves via gynodioecy or the paradioecy-monoecy pathways, the selfing rate has important implications for the extent to which unisexual individuals are favored in populations of hermaphrodites (B. Charlesworth and D. Charlesworth 1978; D. Charlesworth and B. Charlesworth 1978). Our study is the first to explicitly examine the potential for an outcrossing advantage for a monoecious species. The observed difference in outcrossing rate between monoecious and dioecious populations indicates that females or female-biased variants in some monoecious populations are likely to benefit from a substantial increase in the proportion of their ovules that are outcrossed. Models indicate that in the absence of other factors influencing the evolution of dioecy, females, or female-biased variants are favored if the product of the selfing rate and inbreeding depression in hermaphrodites is greater than 0.5 (D. Charlesworth and B. Charlesworth 1978). Indeed, for one population in this study (M-1) these conditions prevail, indicating that selfing and inbreeding depression alone may favor the evolution of female-biased forms in this population. These findings contrast with the argument by Freeman et al. (1997) that the evolution of dioecy from monoecy is not likely to involve inbreeding avoidance.

So far, our arguments have assumed that dioecy is derived from monoecy in *S. latifolia*. There are two lines of evidence that are relevant to this assumption. First, of the 20 species described in Bogin's (1955) monograph of the genus, the vast majority are monoecious. Only two species in the genus appear to be dimorphic for gender: *S. latifolia* (Smith 1894; Wooten 1971; Sarkissian et al. 2001) and *S. lancifolia* (Muenchow 1998). Second, a recent phylogeny indicates that the putative sister taxa of *Sagittaria* is monoecious, suggesting that the ancestral condition for *Sagittaria* is monoecy (Les et al. 1997). Given that most species of *Sagittaria* are monoecious and the ancestral condition for the genus appears to be monoecy, it seems reasonable to assume that dioecy arose from monoecy in *S. latifolia*. Nevertheless, reconstruction of the evolutionary history of sexual systems in *Sagittaria* based on a molecular phylogeny is certainly required to corroborate this assumption.

Our finding that inbreeding avoidance may be important in the evolution of dioecy from monoecy contrasts with previous research on *S. latifolia*. Delesalle and Muenchow (1992) argued that, in spite of opportunities for selfing in "mon-

oecious individuals'' of *S. latifolia*, inbreeding depression may not be strong enough to favor the evolution of unisexuality. Instead, based on observations of preferential weevil herbivory of male flowers in *S. latifolia*, Muenchow and Delesalle (1992) argue that there has been selection for male-biased individuals and that gender dimorphism in *Sagittaria* is likely to have become established via the androdioecy pathway (Muenchow and Delesalle 1992; Muenchow 1998). However, as discussed above, their experimental comparisons of selfed and outcrossed progeny in *S. latifolia* may have underestimated the true magnitude of inbreeding depression in this species. Moreover, in monoecious populations gender expression is strongly size dependent, potentially obscuring the influence of all but complete sterility mutations on allocation to male sex function (Sarkissian et al. 2001). Finally, the likelihood that male-biased individuals will have a fitness advantage in populations of hermaphrodites is inversely related to the selfing rate (Lloyd 1975b; B. Charlesworth and D. Charlesworth 1978; Charlesworth 1984). This is because selfing in hermaphrodites reduces opportunities for males to sire seed in the population. Therefore, size-dependent gender expression and substantial selfing in monoecious population seem likely to reduce the probability that male-biased individuals could establish and spread in monoecious populations of *S. latifolia*.

In this study, we provide evidence that the avoidance of inbreeding is likely to have influenced the evolution of dioecy from monoecy in a clonal, generalist-pollinated species. Indeed, the association between dioecy, large plant size, and generalist modes of pollination is not uncommon (reviewed in Sakai and Weller 1999), indicating that these features may often result in selection for unisexuality. In *S. latifolia*, the unusual existence of conspecific populations that differ in sexual system in the same geographical area allowed us to infer which factors may have been involved in the transition from monoecy to dioecy. Such comparisons help to minimize the influence of phylogenetic divergence that often confound comparative studies of contrasting sexual systems at higher levels in the genealogical hierarchy.

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