



# The role of hybridization in the evolution of sexual system diversity in a clonal, aquatic plant

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The stable coexistence within populations of females, males, and hermaphrodites (subdioecy) is enigmatic because theoretical models indicate that maintenance of this sexual system involves highly restricted conditions. Subdioecy is more commonly interpreted as a transitory stage along the gynodioecious pathway from hermaphroditism to dioecy. The widespread, North American, aquatic plant *Sagittaria latifolia* is largely composed of monoecious or dioecious populations; however, subdioecious populations with high frequencies of hermaphrodites (mean frequency = 0.50) characterize the northern range boundary of dioecy in eastern North America. We investigated two hypotheses for the origin of subdioecy in this region. Using polymorphic microsatellite loci, we evaluated whether subdioecy arises through selection on standing genetic variation for male sex inconstancy in dioecious populations, or results from hybridization between monoecious and dioecious populations. We found evidence for both pathways to subdioecy, although hybridization was the more common mechanism, with genetic evidence of admixture in nine of 14 subdioecious populations examined. Hybridization has also played a role in the origin of androdioecious populations in *S. latifolia*, a mechanism not often considered in the evolution of this rare sexual system. Our study demonstrates how hybridization has the potential to play a role in the diversification of plant sexual systems.

**KEY WORDS:** Dioecy, gender strategies, hybridization, monoecy, *Sagittaria*.

The most common sexual condition in most lineages of flowering plants is gender monomorphism, in which individuals in a population transmit genes to the next generation through female and male function. In contrast, dioecy, in which individuals function primarily as either females or males, occurs in only ~6–7% of angiosperm species and 43% of families, but the sexual polymorphism has evolved repeatedly from hermaphroditism with numerous independent origins (Renner and Ricklefs 1995; Renner 2014). The evolution of dioecy has traditionally been envisioned as a unidirectional and irreversible pathway from hermaphroditism (Bull and Charnov 1985). However, this is not always the case and reversions from dioecy and other forms of gender dimorphism to hermaphroditism are increasingly reported (Lloyd 1975a; Sytsma et al. 1991; Barrett 2002; Pan-

nell 2002; Ehlers and Bataillon 2007; Delph 2009; Schaefer and Renner 2010; Alonso and Herrera 2011; Crossman and Charlesworth 2014). Indeed, gender variation in some species can be near continuous, with numerous intermediate conditions between hermaphroditism and dioecy (Lloyd 1979, 1980; Lloyd and Bawa 1984; Yakimowski and Barrett 2014a). Plant species that display a wide range of gender strategies can provide novel insights into the evolutionary origins of plant sexual diversity, an approach we use here.

Since Darwin (1877), the origin of subdioecy—populations containing females, males, and hermaphrodites—has generally been assumed to arise from evolutionary processes operating within gynodioecious (females and hermaphrodites) and dioecious lineages. The origin of dioecy is frequently thought

to evolve through the “gynodioecy pathway” (Lloyd 1975b; Charlesworth and Charlesworth 1978a; Charlesworth 1999; Spigler and Ashman 2011). Accordingly, females are able to invade hermaphroditic populations when the product of the selfing rate ( $s$ ) and inbreeding depression ( $\delta$ ) is  $>0.5$ . This is then followed by the gradual evolution of male-biased sex allocation and female sterility in hermaphrodites. At this stage in the evolutionary pathway, populations are referred to as subdioecious because they contain three sex phenotypes. A characteristic feature of this genetic pathway is the occurrence in subdioecious populations of plastic sex expression in males, but not females (Ehlers and Bataillon 2007). Following Lloyd (1976), these hermaphrodite plants are termed “inconstant males” and obtain the majority of their fitness through male function (Lloyd 1976; Delph and Lloyd 1991; Barrett et al. 1999; Delph and Wolf 2005; Ashman 2006; Ehlers and Bataillon 2007). Therefore, the observation of populations containing a low frequency (e.g.,  $<10\%$ ) of hermaphrodites (inconstant males) in numerous dioecious species is usually interpreted as a legacy of the gynodioecy pathway (Barrett 1992; Ashman 2006). This hypothesis envisions that the small number of inconstant males in predominantly dioecious populations represents “stalled” variation in populations that have failed to evolve to “pure” dioecy, in which all individuals are strictly unisexual.

A low frequency of conditionally expressed hermaphroditism may be relatively easy to maintain in gender dimorphic populations because the production of a small number of ovules by individuals that gain fitness primarily via pollen is unlikely to compromise male fitness significantly. However, in the context of the gynodioecy pathway it is more difficult to explain the occurrence of high frequencies of hermaphrodites because once pure males evolve they should exhibit higher fitness than hermaphrodites (Charlesworth and Charlesworth 1978a). Nevertheless, hypotheses invoking selection on hermaphroditism have been suggested in this situation. For example, male inconstancy and plastic gender allocation (Delph and Wolf 2005) could be maintained through selection for reproductive assurance during frequent episodes of extinction and recolonization, particularly in a metapopulation (Pannell 1997; Ashman 2006). Alternatively, any decline in female frequency associated with environmental gradients involving resource stress could also select for an increase in hermaphrodite frequency, owing to their reduced investment in seed production compared to females (Yakimowski and Barrett 2014a). Because each of these processes to explain the coexistence of the three sex phenotypes occur within dioecious populations containing inconstant males, we refer to them as involving the “male inconstancy hypothesis” for the origin of subdioecy.

An alternative novel explanation for the origin of subdioecy involves hybridization between hermaphroditic and dioecious populations. We refer to this as the “hybridization hypothesis” and it is novel because despite the plethora of theoretical models con-

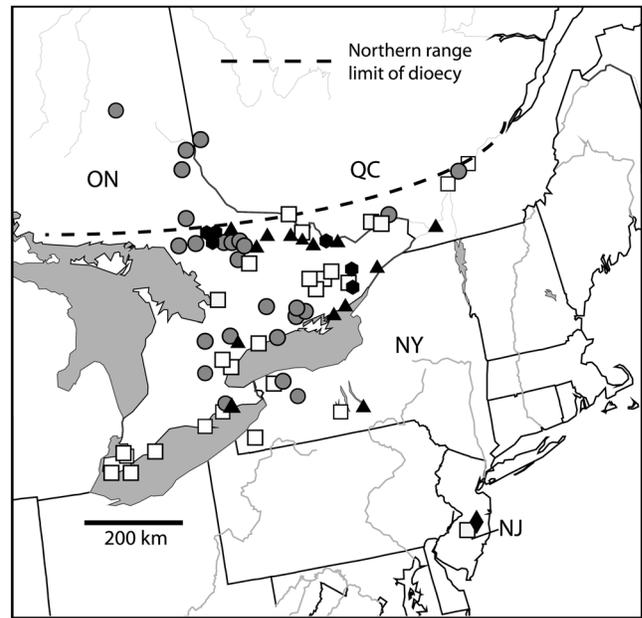
cerning the evolution of gender dimorphism in plants (e.g., Lewis 1941; Ross 1970; Lloyd 1982; Pannell 1997; Charlesworth 1999; Wolf and Takebayashi 2004; Crossman and Charlesworth 2014), hybridization has not been considered in models as a source of sex determining genes promoting sexual system transitions. The potential for hybridization between hermaphroditic and dioecious populations depends on extrinsic and intrinsic factors affecting the degree of reproductive isolation between populations with contrasting sexual systems. However, given the weak isolating mechanisms in many plant species (Grant 1981; Coyne and Orr 2004), this pathway to subdioecy is certainly plausible. Indeed, hybridization between species with contrasting sexual systems has been invoked to explain the origin of androdioecy (males and hermaphrodites) in *Mercurialis* (Pannell et al. 2008). For species with gender monomorphic and dimorphic populations (see Ashman 2006), hybridization between sexual systems could play a role in generating subdioecious populations, especially in cases where populations with contrasting sexual systems are interfertile and occur in close geographical proximity.

*Sagittaria latifolia* (Alismataceae) is a clonal, emergent aquatic that is widely distributed across a variety of wetland habitats in North America (Bogin 1955). In a survey of 116 populations in the northern portion of eastern North America, the majority ( $\sim 2/3$ ) could be classified as either monoecious (hermaphrodite plants with separate female and male flowers) or dioecious (Yakimowski and Barrett 2014a). However, sexual-system variation was near-continuous and one-third of populations exhibited a mixture of sex phenotypes (hereafter referred to as “mixed populations”), with hermaphrodites ranging in frequency from 11 to 79% in combination with females and/or males (see Fig. 2 in Yakimowski and Barrett 2014a). Most (14) of the mixed (20) populations in this survey were subdioecious. Despite the variation in sex phenotype distributions, crossing experiments indicate that sex phenotype segregation supports a Mendelian model of sex determination (Dorken and Barrett 2004b). The model involves a female sterility locus ( $Su^F$ ) at which the dominant allele suppresses female function. In contrast, at the male sterility locus ( $S^M$ ), the dominant allele promotes male function. Plants from monoecious populations possess a distinct homozygous sex-determining genotype,  $Su^f S^M / Su^f S^M$ , from which pure males or pure females cannot segregate. In dioecious populations, females are homozygous recessive at both loci,  $Su^f S^m / Su^f S^m$ , and there are two male genotypes: pure males,  $Su^F S^M / Su^F S^m$  and inconstant males,  $Su^F S^M / Su^f S^m$ , heterozygous at the male sterility locus. The adherence of sex phenotype segregation in crosses to this two-locus two allele model is consistent with Charlesworth and Guttman’s (1999) genetic model of the gynodioecy pathway to dioecy in which gender is determined by two strongly linked genes, rather than many genes of small effect, as expected for the monoecy pathway to dioecy (Charlesworth and Charlesworth

1978b; Charlesworth 1999). This pattern of inheritance and the absence of female inconstancy supports the hypothesis that dioecy has evolved via the gynodioecious pathway in *S. latifolia*.

Monoecious and dioecious populations of *S. latifolia* occur in contrasting wetland habitats and are differentiated in a range of life-history traits, including flowering time (Dorken & Barrett, 2003). These differences likely contribute toward their reproductive isolation, as indicated by genetic differentiation between monoecious and dioecious populations at molecular markers (Dorken et al. 2002; Dorken and Barrett 2004a). Yet, individuals from monoecious and dioecious population are fully inter-fertile under glasshouse conditions (Dorken and Barrett 2004b), raising the possibility of hybridization between the sexual systems under field conditions. This could involve gene flow via pollen or seeds between populations of contrasting sexual system, or by a breakdown in ecological isolation permitting coexistence of plants from monoecious and dioecious populations. The combination of sexual system polymorphism and genetic differentiation between monoecy and dioecy in *S. latifolia* therefore provides a rare opportunity for investigating the origins of subdioecy. Haplotypes and/or allele frequencies of monoecious and dioecious populations can be used to estimate genetic admixture of the two sexual system lineages and directly test the hybridization hypothesis.

Here, we investigate patterns of cpDNA (chloroplast DNA) and microsatellite (SSR - simple sequence repeat) variation in populations of *S. latifolia* sampled from northeastern North America comprising diverse sexual systems. The principal objective of our study was to distinguish between the “male inconstancy” and “hybridization” hypotheses for the origin of subdioecious populations. However, our survey also revealed the occurrence of androdioecious and gynodioecious populations and we also address their origins using the population genetic data. Our study involved the following specific questions: (1) Do subdioecious populations cluster genetically with dioecious populations as predicted by the male-inconstancy hypothesis, or alternatively do subdioecious populations exhibit evidence of genetic admixture consistent with the hybridization hypothesis? (2) What types of hybrid crosses are likely to have given rise to the patterns of assignment we observe? The ability to assign admixed individuals to particular hybrid classes increases support for hybridization and decreases the possibility that “hybrids” simply represent individuals for which assignment is uncertain. (3) Is there evidence that androdioecious and gynodioecious populations have also arisen by hybridization, and how might this be influenced by the sex determining genotypes produced by crossing? The occurrence in *S. latifolia* of populations with sex phenotype distributions representing five recognized sexual systems (Yakimowski and Barrett 2014a) provides an exceptional opportunity to determine the evolutionary pathways responsible for their origin.



**Figure 1.** The geographical locations in northeastern North America of the 25 monoecious (gray circles), 27 dioecious (white squares), 13 subdioecious (black triangles), six androdioecious (black hexagons), and one gynodioecious (diamond) populations of the aquatic plant *Sagittaria latifolia* investigated in this study using cpDNA and SSR markers.

## Methods

### MOLECULAR MARKER SURVEYS

We conducted a survey of cpDNA haplotypes, which in the sampled range of *S. latifolia* (Fig. 1) are known to differentiate monoecious (M haplotype) and dioecious (D haplotype) populations (Dorken and Barrett 2004a). We surveyed 66 populations with contrasting sex phenotype ratios for these two chloroplast haplotypes to compare the genetic composition of populations. Our sample comprised 24 monoecious populations, 22 dioecious populations ranging in hermaphrodite frequency from 0 to 8% (mean = 2.2%), and 20 “mixed” populations (containing unisexuals and hermaphrodites with frequencies of >10%). Of the 20 mixed populations, 14 were subdioecious and six were androdioecious (males and hermaphrodites). A major objective of our study was to determine whether individuals occurring in subdioecious populations possessed the dioecious haplotype, as predicted by the sex inconstancy hypothesis, or alternatively whether both haplotypes M and D co-occur within mixed populations, indicating the potential for genetic admixture between dioecious and monoecious populations.

We isolated total DNA from leaf tissue dried and stored in silica gel using a CTAB protocol outlined in Yakimowski and Eckert (2008). Further modifications included grinding 5 mg dried leaf tissue with two glass beads in a 2 mL microtube for 1 min at

30 beats/sec using a Retsch 301 tissue grinder. We extended cell lysis incubation time to 3 h to improve DNA quality and DNA concentration was estimated using a BioPhotometer 6131 (Eppendorf). We assayed variation for one cpDNA fragment (TFef) and restriction enzyme combination (HinfI; Taberlet et al. 1991) differentiating the two cpDNA haplotypes (Dorken and Barrett 2004a). We assayed a total of 645 individuals, including an average of five individuals from monoecious and dioecious populations and 25 individuals from mixed populations. We performed 50  $\mu$ L polymerase chain reaction (PCR) reactions using a PTC-220 DNA Engine DyadTM Peltier Thermal Cycler under conditions described in Dorken and Barrett (2004a). Restriction reactions were composed of 5  $\mu$ L PCR product, 1  $\mu$ L 10 $\times$  enzyme buffer, and one unit of restriction enzyme. These reactions were incubated at 37°C for 16 h. We resolved polymorphisms on 1.6% agarose gel run at 100 V for 2.5 h.

To evaluate evidence for genetic admixture within individuals, we assayed codominant genetic variation using 11 polymorphic microsatellite loci in 42 populations, 36 of which are a subset of those described above. This sample comprised 10 monoecious populations (276 individuals; mean = 23.9 per population), 13 dioecious populations ranging in hermaphrodite frequency from 0 to 8.4% (mean 5.2%; 314 individuals; mean = 23.6 per population), and 19 mixed populations (637 individuals; mean = 31.3 per population; Fig. 1). Of the 19 mixed populations 13 were subdioecious, five were androdioecious, and one was gynodioecious. PCR reactions and conditions followed Yakimowski et al. (2009).

#### ASSIGNMENT ANALYSES AND ESTIMATING HYBRIDIZATION

We conducted model-based assignment tests for all individuals from the 42 populations sampled for microsatellite variation using Bayesian clustering software *Structure* (Pritchard et al. 2000; Falush et al. 2003). This procedure assigns a proportion ( $q$ ) of an individual's "genome" to  $K$  populations, maximizing Hardy-Weinberg and linkage equilibrium. All of the *Structure* runs were executed without any *a priori* information regarding the population of origin. Simulation models allowed for admixture of genetic clusters within individuals, and assumed that allele frequencies were correlated among clusters, which gives accurate assignment of individuals in closely related populations (Pritchard et al. 2000). We used a burn-in length of 25,000, which was sufficient for values of  $\alpha$  to converge, and then ran the simulation 1,000,000 times to obtain accurate parameter estimates. We used the option for *Structure* to collect information on the distribution of each estimate of  $q$ , which provides a probability interval, a Bayesian analogue of a confidence interval, for each  $q$ .

Because previous studies demonstrated genetic differentiation between monoecious and dioecious populations of *S. latifolia* (Dorken et al. 2002; Dorken and Barrett 2004a), we had an *a priori*

interest in testing  $K = 2$  to determine whether individuals from monoecious and dioecious populations could be reliably assigned to two clusters based only on their genotypes, and whether these individuals could be distinguished from individuals that were genetically admixed. We also examined  $K > 2$  to determine the "true"  $K$  by running 20 simulations for each  $K = 1-10$  and identified the  $K$  associated with the lowest SD among runs (Evanno et al. 2005).

Using *Structure*, we defined an individual as "pure" (free of genetic admixture) if  $q$  was 95% or greater for a single cluster. Estimates of  $q$  can also be used as a hybrid index and therefore individuals exhibiting  $0.5 < q < 0.95$  can be considered potential hybrids. However, *Structure* also assigns intermediate  $q$  values if there is uncertainty associated with the assignment of an individual. Therefore, to further distinguish potential hybrids we employed the Bayesian model-based clustering program *NewHybrids* (Anderson and Thompson 2002), which estimates the posterior probability that admixed individuals belong to distinct genealogical classes through Markov chain Monte Carlo (MCMC) methods. We used the default set of classes that includes the following: (1) pure monoecious, (2) pure dioecious, (3) F<sub>1</sub> hybrids, (4) F<sub>2</sub> hybrids, (5) backcross to monoecious, and (6) backcross to dioecious. We also summed the probabilities for (2) to (6) to assign individuals to a more general hybrid category following Field et al. (2010). We ran the software with a burn-in of 25,000 and >1,000,000 generations and used "Jeffrey's like priors." We used 50% as a threshold for assigning an individual to a particular hybrid class following Vähä and Primmer (2006). In monoecious and dioecious populations, 97% of the 29 probable hybrids detected by this method corresponded to *Structure*-assigned hybrids—a single mismatch occurred in monoecious population OTK-NY. In mixed populations, 87% of the probable hybrids corresponded to *Structure*-assigned hybrids—the mismatches occurred in two populations (one subdioecious, one androdioecious) in which eight individuals in each were identified as pure monoecious by *Structure*, and as probable hybrids by *NewHybrids*. These individuals were not included in any subsequent analyses of hybrid frequency (see Dryad data for these mismatches).

To test for evidence of the hybrid origins of subdioecy, we calculated the proportion of individuals in each population assigned to any hybrid category using *NewHybrids* and compared this proportion among monoecious, dioecious, and subdioecious populations using a generalized linear model with a binomial distribution in *R* version 2.8.1 (2013). We also calculated the mean frequency of hybrids  $\pm$  SD in monoecious and dioecious populations. We considered mixed populations falling within this mean  $\pm$  SD interval as supporting the male inconstancy hypothesis for the origin of subdioecy. In contrast, populations exhibiting frequencies of hybrids higher than the upper limit of this

interval were considered to support the hybridization hypothesis. We performed this analysis with both the *Structure* and *NewHybrids* estimates of hybrid frequency.

## Results

### cpDNA HAPLOTYPE STRUCTURE

Monoecious and dioecious populations of *S. latifolia* were largely associated with different cpDNA haplotypes. All 22 dioecious populations exhibited haplotype D (Fig. 2A), and all but three of the 25 monoecious populations were composed of haplotype M. One monoecious population contained a mixture of haplotypes M and D, and two populations were composed of haplotype D, indicating that they were probably founded by inconstant males from dioecious populations and therefore represent reversions from dioecy to monoecy (Fig. 2C). Mixed populations (hermaphrodite frequencies = 11–79%) exhibited a much greater variety of cpDNA haplotype structure (Fig. 2B). Of the 14 subdioecious populations, five contained only haplotype D and nine contained a mixture of individuals with haplotypes D and M. Among subdioecious populations containing both cpDNA haplotypes, the mean frequencies of haplotypes D and M were 0.78 (range 0.08–1.0) and 0.22 (range 0.0–0.92), respectively.

### GENETIC DIFFERENTIATION OF SEXUAL SYSTEMS AND EVIDENCE FOR HYBRIDIZATION

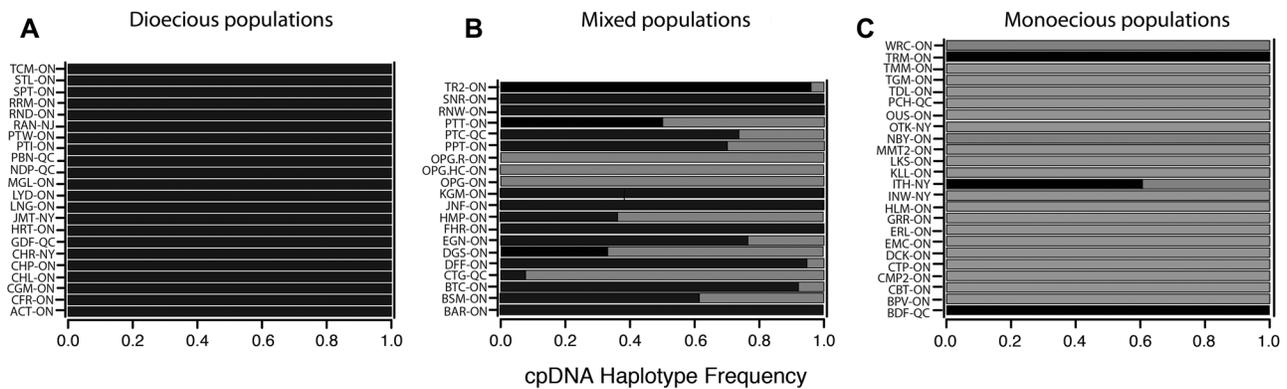
The amount of differentiation in SSR markers between monoecious and dioecious populations was sufficient to distinguish individuals of the two sexual systems using assignment-based *Structure*. For  $K = 2$ , we found that among the 273 individuals sampled from 10 monoecious populations, 97.2% of their genomes were on average assigned by *Structure* to cluster M (Fig. 3A—blue bars), and for the 317 individuals sampled from 13 dioecious populations, 96.1% of their genomes were assigned to cluster D (Fig. 3B—red bars). Individuals that had 95% or more of their genome assigned to one genetic cluster or the other were considered as “pure” with no evidence for admixture. On average, 89.2% (range = 65.2–100%) of individuals in monoecious populations could be unambiguously assigned to cluster M, and in dioecious populations 82.2% (range = 52.3–100%) could be assigned to cluster D. No individuals in monoecious or dioecious populations were assigned as pure D or M, respectively. For most individuals in monoecious (97%) and dioecious (93%) populations, the 90% CI for these assignments do not overlap the 50% line (Fig. S2). Therefore, the vast majority of individuals occurring in dioecious and monoecious populations can be assigned with high confidence to clusters D and M, respectively, (Fig. 3A, B). Finally, as expected based on prior genetic evidence,  $K = 2$  was found to be the “true” number of genetic clusters (Fig. S1).

The patterns of genetic variation were much more complex for the 19 mixed populations of *S. latifolia* included in the *Structure* assignment analysis. Among the 505 individuals sampled from 13 subdioecious populations, 21.8% of the genome of individuals was on average assigned to cluster M and 78.2% was assigned to cluster D. On average, 7.5% of individuals in subdioecious populations were assigned as pure M, 49.1% of individuals were assigned as pure D, and 43.3% of individuals exhibited admixture of clusters M and D. The percentage of admixed individuals (40.2%) in the 13 subdioecious populations was significantly higher than the percentage (14.1%) observed in dioecious and monoecious populations (GLM:  $z$ -value =  $-9.4$ ,  $df = 35$ ,  $P < 0.0001$ ). The percentage of admixed individuals, as estimated more conservatively by *NewHybrids*, was also significantly higher in subdioecious populations than in monoecious or dioecious populations (23.4 vs. 5.4% respectively; GLM:  $z$ -value =  $-6.5$ ,  $df = 35$ ,  $P < 0.0001$ ). Adding latitude to the model did not improve the overall fit of the model for the *Structure* estimate of hybrid frequency.

Of the 13 subdioecious populations examined, nine were composed of pure D (mean = 55.7%) and admixed (mean = 38.1%) individuals, and no pure M individuals were evident in the populations. Of these nine populations, three (FHR-ON, KGM-ON, TR2-ON) exhibited frequencies of admixed individuals as estimated by *Structure* that fell within the range observed in monoecious and dioecious populations (mean =  $14.8\% \pm 14.0$  SD), whereas the frequency of admixed individuals in the remaining six populations exceeded this range of values. Four of the subdioecious populations were composed of pure M (mean = 26.5%), pure D (mean = 33.6%), and admixed (mean = 39.9%) individuals. Of these four populations, only one (PTT-ON) exhibited levels of admixture within the range of monoecious and dioecious populations; the other three populations exhibited frequencies of admixed individuals greater than occurred in monoecious and dioecious populations (Fig. 3C). In summary, among the 13 subdioecious populations nine exhibited patterns of variation consistent with the hybridization hypothesis, one population exhibited a mixture of monoecious and dioecious plants but with a low frequency of hybrid individuals, and the remaining three populations exhibited patterns more consistent with the male-inconstancy hypothesis.

### ASSIGNMENT OF HYBRID CLASSES

The assignment of pure M and D individuals of *S. latifolia* with *NewHybrids* was consistent with the results from *Structure*. We found that a subset of genetically admixed individuals identified by *Structure* was also identified as hybrid by *NewHybrids*. On average, 29.1% of the admixed individuals in monoecious populations were identified as hybrids (total  $n = 9$  from *NewHybrids* compared with 28 from *Structure*), 27.7% of admixed



**Figure 2.** Chloroplast DNA haplotype frequencies for 66 populations of *Sagittaria latifolia* in northeastern North America. (A) Twenty-two dioecious populations composed of females and males, with hermaphrodite frequency 0–10%, (B) 20 mixed populations, and (C) 24 monoecious populations are composed predominantly (80–100%) of the hermaphrodite phenotype. Black bars represent the proportion of samples associated with haplotype D and gray bars represent the proportion of samples associated with haplotype M. Within each column (A–C) populations are sorted alphabetically from bottom to top. Sex phenotype distribution data for each of these populations can be found in Figure 2 and Appendix S1 of Yakimowski and Barrett (2014a).

individuals in dioecious populations ( $n = 20$  of 55) were identified as hybrids, and 46.4% of admixed individuals in subdioecious populations ( $n = 90$  of 194) were identified as hybrids (Fig. 3C). This translates to an average of 0.9, 1.5, and 6.9 individuals identified as hybrids in monoecious, dioecious, and subdioecious populations, respectively. Averaged across the 90 hybrids identified in subdioecious populations using *NewHybrids*, the posterior probability of hybrids being  $F_2$  plants was highest (0.67), the probability of hybrids being backcrosses to dioecious plants was much lower (0.12), and the probabilities of hybrids being  $F_1$  plants (0.0007) or backcrosses to monoecious plants (0.0007) were both negligible. Of the nine subdioecious populations identified as hybrid based on *Structure* estimates of genetically admixed individuals, seven also exhibited a frequency of *NewHybrids* greater than the range observed in monoecious and dioecious populations (mean =  $4.8\% \pm 8.4$  SD).

#### ANDRODIOECIOUS AND GYNODIOECIOUS POPULATIONS

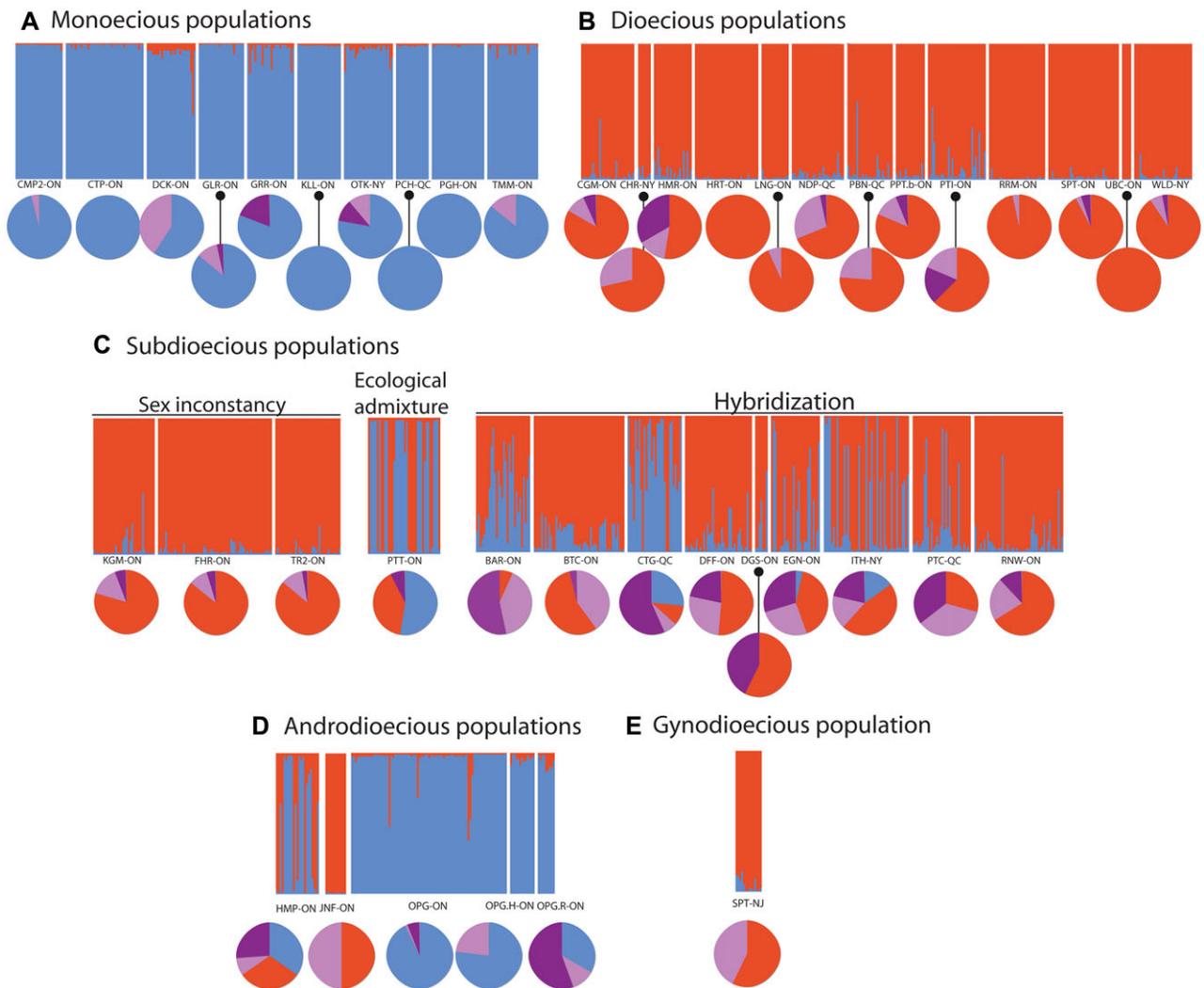
Our study was primarily designed to understand the origin of subdioecious populations; however, *S. latifolia* exhibits near-continuous gender variation from monoecy to dioecy at the northern portion of its geographical range, including androdioecious and gynodioecious populations (Yakimowski and Barrett 2014a, Fig. 2). Thus, we included these populations in our assignment analyses to determine which sexual systems (monoecy vs. dioecy) they clustered with and whether they were of hybrid origin.

Among the six androdioecious populations, one contained only cpDNA haplotype D, two contained a mixture of cpDNA haplotypes D and M, and three contained only cpDNA haplotype M. Using SSR markers, one of five androdioecious populations

was found to contain only pure D individuals (JNF-ON). The other four androdioecious populations were composed of an average of 59.4% pure M individuals, 7.6% pure D, and 32.9% admixed individuals (Fig. 3E). Of these four populations, two (HMP-ON and OPG.R-ON) exhibited a frequency of admixture greater than the range observed in monoecious and dioecious populations using both *Structure* and *NewHybrid* estimates (Fig. 3D). The 11 *NewHybrids* were on average assigned a 0.89 probability of being  $F_2$  hybrids. We were unsuccessful in obtaining the cpDNA data from the single gynodioecious population (SPT-NJ) despite many trials, a technical difficulty unique to this population. Using SSR markers, 57.1% of the individuals sampled were assigned to cluster D, and although 42.9% exhibited genetic admixture none were identified as hybrids by *NewHybrids* (Fig. 3E).

#### Discussion

Since Darwin's (1877) seminal work on plant sexual systems, subdioecy has most often been viewed as a transient condition associated with the evolution of dioecy. However, several lines of evidence indicate that the subdioecious populations we examined in *S. latifolia* are unlikely to represent an intermediate stage in the gynodioecious pathway to dioecy. The populations were characterized by a high frequency of hermaphrodite plants (mean frequency = 0.50), unlike the low frequency of male inconstancy typical of most dioecious and subdioecious populations. It is therefore unlikely that hermaphroditism in these populations represents "stalled variation" associated with the gynodioecious pathway (Ashman 2006). Significantly, subdioecious populations of *S. latifolia* have a highly clustered geographic distribution, occurring commonly at the northeastern range limit of dioecy. Given the widespread distribution of both sexual systems in eastern and



**Figure 3.** *Structure* and *NewHybrids* analyses of individuals from (A) 10 monoecious, (B) 13 dioecious, (C) 13 subdioecious, (D) five androdioecious, and (E) one gynodioecious population of *Sagittaria latifolia* from northeastern North America. Each vertical bar represents the proportional assignment of an individual's genome by *Structure* ( $K = 2$ ) to cluster D (red) and cluster M (blue). Pie charts indicate the proportion of individuals assigned by *Structure* as pure D (red), pure M (blue), and admixed (mauve). Dark purple portions of the pie charts indicate the proportion of admixed individuals that were verified as hybrids by *NewHybrids*. Subdioecious populations are arranged into groups of populations that support the "male inconstancy" and "hybridization" hypotheses; one population is labeled "ecological admixture" as it fell into neither category but contained both pure D and M individuals without evidence for genetic admixture.

western North America, this is an unlikely focal area for a transition from monoecy to dioecy. Moreover, none of the subdioecious populations we examined in this study were assigned primarily to the same genetic cluster as monoecious populations, as would be predicted if dioecy had arisen relatively recently from monoecy and populations represented various stages associated with the transition to dioecy. Rather, our marker-based evidence indicates the opposite polarity and supports the hypothesis that subdioecious populations are largely derived from dioecy, arising by two distinct mechanisms.

Our study evaluated evidence for two alternative hypotheses—the male inconstancy and hybridization hypotheses—for

the origin of subdioecious populations in *S. latifolia*. We found strong support for genetic admixture in 9 of the 13 subdioecious populations that we examined. Of the remaining populations, one population contained pure monoecious and dioecious individuals, but no evidence of an elevated frequency of hybrid individuals. In three subdioecious populations, the frequency of admixed individuals fell within the range observed in monoecious and dioecious populations. We begin our discussion by examining the contributions of hybridization and male inconstancy to the evolution of subdioecy in *S. latifolia*. We consider how geographical factors and local climatic selection may play a role in the breakdown of reproductive isolation between monoecious and dioecious

populations, and we also examine the extent to which subdioecy may be maintained in populations over longer time scales. We conclude by discussing the implications of our findings for the evolution of polymorphic sexual systems in plants.

### ORIGIN OF SUBDIOECY BY HYBRIDIZATION

In ~70% of the subdioecious populations we examined, individuals exhibited patterns of genetic admixture consistent with the occurrence of hybridization between individuals from monoecious and dioecious populations. Several independent lines of evidence support the hybridization hypothesis: (1) None of the monoecious or dioecious populations exhibited the co-occurrence of pure monoecious and dioecious individuals within them. This result is in accord with earlier work demonstrating the ecological and genetic differentiation of monoecious and dioecious populations in eastern North America (Dorken et al. 2002; Dorken and Barrett 2004a). (2) In contrast, five of the 13 subdioecious populations were associated with the co-occurrence of pure M and D individuals, and four of these exhibited mean frequencies of genetically admixed individuals significantly higher than in monoecious or dioecious populations. This demonstrates that the co-existence of hermaphrodite and unisexual individuals originating from monoecious and dioecious populations, respectively, promotes hybridization. (3) Overall, the mean frequency of genetically admixed individuals was significantly higher in subdioecious than in monoecious or dioecious populations. (4) Modeling of genealogical hybrid classes indicated that the majority of hybrids assigned by *NewHybrids* have an ~70% chance of being  $F_2$  hybrids, and on average an ~10% chance of being a backcross to a dioecious parental. Greater marker resolution would be needed to improve assignments for a larger number of individuals (Vähä and Primmer 2006); however, in all but one population with a high frequency of admixed individuals, one to eight individuals had at least a 90% probability of being an  $F_2$  hybrid. These results suggest that even if the frequency of admixed individuals is an overestimate of hybridity, there are individuals with a very high probability of being true hybrids in mixed populations. Our study therefore provides the first evidence for the occurrence of hybridization under field conditions between monoecious and dioecious populations of *S. latifolia*.

We detected pure monoecious and dioecious plants coexisting in five subdioecious populations (four hybrid populations, and one population with a mixture of monoecious and dioecious individuals without genetic admixture), a pattern that we did not detect in any of the monoecious or dioecious populations. This raises the question of what ecological conditions favor the breakdown of habitat isolation between monoecious and dioecious populations leading to hybridization. One possibility is that at the northern range limit of dioecy intermediate or variable ecological conditions, perhaps involving greater disturbance, may promote coex-

istence of plants from both sexual systems, although currently we have no direct evidence bearing on this point. It might also be predicted that dioecious populations would be more prone to invasion by hermaphrodites, given the ease with which monoecious populations can colonize a variety of disturbed wetland sites (Dorken and Barrett 2003, 2004a). Both the predominance of the dioecious “genome” in subdioecious populations, and the lower frequency of pure D individuals in dioecious populations relative to the frequency of pure M individuals in monoecious populations are consistent with this hypothesis. Detailed comparisons of habitat conditions in monoecious, dioecious, and subdioecious populations would be needed to determine if the prevalence of subdioecious populations in the region we studied is associated with changes in ecology with latitude.

The breakdown of reproductive isolation may also be associated with a convergence in the life history and phenology of populations of the two sexual systems toward the range limit of dioecy. The majority of hybrid populations occur very near the northern range limit of dioecy, with only two more southerly populations (ITH-NY and DFF-ON). At this range limit, the growing season is relatively short and this influences the phenology of populations. More southerly dioecious populations grow larger and flower later compared to monoecious populations at the same latitude (Dorken and Barrett 2003). Selection at northern range limits may therefore favor the evolution of a more “monoecious-like” life-history strategy in dioecious populations, resulting in greater overlap in flowering time and possibly convergence in habitat requirements. Field observations of flowering times between monoecious and dioecious populations in northern populations are consistent with this hypothesis (S. C. H. Barrett unpubl. obs.). In addition, the fact that monoecious populations extend further north than dioecious populations in eastern North America (Dorken and Barrett 2004a) supports the view that the life history of monoecious populations may be better able to cope with a shorter growing season, where there is often a premium on flowering earlier at a smaller size and maturing seed before frosts occur in September (Colautti and Barrett 2013). Future studies of variation in life-history strategy and flowering phenology of monoecious, dioecious, and mixed populations along a latitudinal gradient could further assess this “life-history convergence” hypothesis.

Hybridization without contemporary coexistence of pure monoecious and dioecious individuals within a population was also revealed by our study. Five of nine subdioecious populations were composed of only pure D and admixed individuals. This pattern suggests that hybridization could have occurred as a result of pollen dispersal from monoecious to dioecious populations, which would occur more frequently if flowering times overlapped more strongly with increasing latitude. The numerous generalist insect pollinators of *S. latifolia* (Glaetli and Barrett 2008) could readily promote pollen dispersal among populations of this

species. One of the five populations only contained cpDNA haplotype D (Fig. 2), a pattern consistent with this scenario. Four of the five populations contained both cpDNA haplotypes, suggesting that monoecious plants were once present in these populations and were maternal parents to hybrid progeny, but that monoecious parentals were subsequently outcompeted.

#### ORIGIN OF SUBDIOECY FROM MALE INCONSTANCY

Dioecious populations with low levels of male sex inconstancy (<10%) are found scattered throughout the geographical range of *S. latifolia* in North America. These populations exhibit a low level of standing variation for male inconstancy, probably a legacy of the gynodioecy pathway involved in the transition from monoecy to dioecy (Dorken and Barrett 2004b). In our survey, all of these populations clustered for both cpDNA and SSR markers with dioecious populations containing only unisexual plants. What maintains the low levels of male sex inconstancy in some dioecious populations of *S. latifolia* but not others is unclear, given the lack of any obvious geographical or ecological pattern to the distribution of populations, but this situation is typical of many dioecious species and warrants further investigation.

Our analysis of SSR variation indicated that three of the 13 subdioecious populations exhibited very low levels of hybridization that could not be distinguished in terms of admixture from patterns observed in monoecious or dioecious populations. This finding is consistent with the hypothesis that subdioecy in these populations has arisen from standing genetic variation in male inconstancy within dioecious populations, perhaps resulting from selection favoring an increase in hermaphrodite frequency. However, we cannot completely rule out the occurrence of past hybridization events in contributing to this pattern because hypervariable SSR markers evolve rapidly, possibly eroding the signature of earlier hybridization events. Indeed, there is some evidence that this may have occurred in subdioecious population, TR2-ON. This population clustered with dioecious populations for SSR markers, but contained a single plant with cpDNA haplotype M, possibly indicating past hybridization. If these three populations, or their ancestors, did experience hybridization historically, it would suggest that high hermaphrodite frequencies can be maintained by selection in subdioecious populations over relatively long time periods.

#### HYBRIDIZATION AND SEXUAL SYSTEM DIVERSITY

Although polyploidization is increasingly recognized as an important factor in the diversification of plant sexual systems (Pannell et al. 2004; Obbard et al. 2006; Ashman et al. 2013; Ludwig et al. 2013; Blank et al. 2014; Ekrt and Koutecký 2016), homoploid hybridization between sexual systems has seldom been considered as a mechanism promoting variation in sex ratios and transitions among sexual systems (but see Barr 2004; Pannell et al. 2008). We

have demonstrated that the majority of subdioecious populations of *S. latifolia* examined show evidence of genetic admixture between monoecious and dioecious populations. Although our study exploited intraspecific variation in sexual systems, the possibility of the hybrid origins of subdioecy and other polymorphic sexual systems should not be discounted in other groups where dioecious and hermaphroditic taxa occur in sympatry. For example, *Ecbalium elaterium* and *E. dioicum* (Costich 1995), *Wurmbea dioica* (Case and Barrett 2004), and *Schiedea mannii* and *S. hookeri* (Weller et al. 2001) all possess unisexuals and hermaphrodites that overlap to varying degrees in distribution providing opportunities for the admixture of sex determining genes and the formation of novel combinations of sexual phenotypes.

Our study also suggests that hybridization has the potential to play a role in the origin of other polymorphic sexual systems. For example, hybridization appears to have been involved in the origin of some of the androdioecious populations of *S. latifolia* investigated (e.g., HMP-ON and possibly ORR-ON; Fig. 3D), albeit not as commonly as with subdioecy. Androdioecy is a rare sexual system in plants that is commonly misidentified, especially in species where size-dependent gender modification influences the gender expression of hermaphrodites causing them to function only as male when small. Indeed, this situation occurs in monoecious populations of *S. latifolia* (Sarkissian et al. 2001). However, males and hermaphrodites were of similar size in the androdioecious populations in our study, suggesting these androdioecious populations are not simply monoecious populations exhibiting small, male-functioning plants.

Populations containing male and hermaphrodite plants in *S. latifolia* can originate simply through mating between particular sex-determining hybrid genotypes, resulting in families that are only able to segregate male and hermaphrodite offspring (Table 1 and see Dorken and Barrett 2004b; Barrett et al. 2010). Importantly, hybridization between monoecy and dioecy results in two male-determining genotypes that are not found in dioecious populations and this increases the number of mating combinations that produce seed families containing only males and hermaphrodites. If such genotypes are dispersed to isolated sites where there is restricted gene flow from monoecious and dioecious populations, this could potentially lead to rapid transitions to androdioecy. In contrast, there are only two mating combinations that result in seed families composed of only females and hermaphrodites (Table 1). Neither of these mating combinations involve male genotypes unique to hybrid populations, therefore these crosses could result from dioecious, subdioecious, or hybrid populations. Yet, the single gynodioecious population revealed by our survey seems likely to have been influenced by genetic admixture. Only 57.1% of the individuals in the population could be assigned as pure D in contrast to an average of 82.2% of the individuals in dioecious populations.

**Table 1.** Progeny sex ratio predictions for genotypic mating combinations that produce “androdioecious” (1–6) or “gynodioecious” (7–8) seed families. The mating scenario shown in the first row (bold) would occur by mating between individuals from monoecious and dioecious populations. Subsequent examples (2–6) would first require hybridization between monoecious and dioecious populations, such as the hybrid subdioecious populations identified in this study (Fig. 3), creating two new hybrid male genotypes. These hybrid male genotypes could subsequently mate with females and hermaphrodites to create “androdioecious” seed families and potentially androdioecious populations. Scenarios 7–8 could take place in dioecious or subdioecious populations and do not require hybridization. The sex determination mechanism indicated is that demonstrated for *Sagittaria latifolia* (see Dorken and Barrett 2004b).

	Maternal sex-determining genotype		Paternal sex-determining genotype	Expected offspring ratio
Mating combinations producing “androdioecious” seed families				
(1)	<b>Monoecious</b> <b><math>Su^fS^M/Su^fS^M</math></b>	x	<b>Male</b> <b><math>Su^FS^M/Su^fS^m</math></b>	<b>50% Hermaphrodite <math>Su^fS^M/Su^fS^m</math></b> <b>50% Male <math>Su^fS^M/Su^FS^M</math></b>
(2)	Female $Su^fS^m/Su^fS^m$	x	Hybrid male $Su^fS^M/Su^FS^m$	50% Hermaphrodite $Su^fS^m/Su^fS^m$ 50% Male $Su^fS^m/Su^FS^M$
(3)	Inconstant male $Su^fS^M/Su^fS^m$	x	Hybrid male $Su^fS^M/Su^FS^m$	50% Hermaphrodite $Su^fS^M/Su^fS^M$ , $Su^fS^m/Su^fS^m$ 50% Male $Su^fS^M/Su^FS^M$ , $Su^fS^m/Su^FS^M$
(4)	Inconstant male $Su^fS^M/Su^fS^m$	x	Hybrid male $Su^fS^M/Su^FS^m$	67% Hermaphrodite $Su^fS^M/Su^fS^M$ , $Su^fS^m/Su^fS^m$ 33% Male $Su^fS^M/Su^FS^M$
(5)	Monoecious $Su^fS^M/Su^fS^M$	x	Hybrid male $Su^fS^M/Su^FS^M$	50% Hermaphrodite $Su^fS^M/Su^fS^M$ 50% Male $Su^fS^M/Su^FS^M$
(6)	Monoecious $Su^fS^M/Su^fS^M$	x	Hybrid male $Su^fS^M/Su^FS^m$	50% Hermaphrodite $Su^fS^M/Su^fS^M$ 50% Male $Su^fS^M/Su^FS^m$
Mating combinations producing “gynodioecious” seed families				
(7)	Female $Su^fS^m/Su^fS^m$	x	Inconstant male $Su^fS^M/Su^fS^m$	50% Female $Su^fS^m/Su^fS^m$ 50% Inconstant male $Su^fS^M/Su^fS^m$
(8)	Inconstant male $Su^fS^M/Su^fS^m$	x	Inconstant male $Su^fS^M/Su^fS^m$ (selfing)	75% Hermaphrodite $Su^fS^M/Su^fS^M$ , $Su^fS^M/Su^fS^m$ 25% Female (may not occur) <sup>1</sup>

<sup>1</sup>Selfing of inconstant males resulted in 100% hermaphrodites, rather than the 3:1 ratio of hermaphrodites to females expected from Mendelian segregation (Dorken and Barrett, 2004b). Therefore, selfing of inconstant males is unlikely to be a source of “gynodioecious” seed families.

Our finding that gynodioecious populations are rare relative to androdioecious populations is consistent with the observation that there are far fewer mating combinations resulting in “gynodioecious” than “androdioecious” seed families. This pattern would be reversed in a system with the opposite pattern of heterogametic sex determination in which females are the heterogametic sex rather than males (e.g., *Silene otitis*; Slancarova et al. 2013). The simple Mendelian basis of sex determination in *S. latifolia* (Dorken and Barrett 2004b) can allow the *de novo* origin of populations with different combinations of the three sex phenotypes. This mechanism, aided by dispersal and restricted gene flow, could promote sexual system transitions in other gender dimorphic species, depending on the details of sex determination and the genetic composition of the founder pool.

A final question concerns the long-term maintenance of subdioecy and whether this sexual system is evolutionarily stable. Theoretical models indicate that the stability of subdioecy occurs under rather restricted conditions (Maurice and Fleming

1995; Wolf and Takebayashi 2004; Ehlers and Bataillon 2007; Crossman and Charlesworth 2014). However, in common with most modeling work on sexual system transitions, these models do not generally account for the fact that gender dimorphic species are often long-lived and many are clonal (but see Seger and Eckhart 1996). These features can greatly retard progress to any deterministic equilibrium. In species with such life histories, many populations when sampled are in a nonequilibrium state and sex- or morph-ratio dynamics can be greatly influenced by the genotypes founding populations and the extent of sexual recruitment (Morgan and Barrett 1988; Eckert and Barrett 1995; Field et al. 2013). Indeed, a model incorporating these factors and using the known sex-determining system of *S. latifolia* suggested that many subdioecious populations of this species are likely to be in a nonequilibrium state (Barrett et al. 2010). However, a recent study of fitness components in subdioecious populations of *S. latifolia* indicated that the sex ratio of populations and sex allocation of hermaphrodites were both consistent with the

equilibrium predictions of Lloyd's (1976) model for gynodioecious and subdioecious populations (Yakimowski and Barrett 2014a). Moreover, marker-based studies of clonal growth and sexual recruitment in *S. latifolia* indicate that sexual recruitment may not be as restricted as our previous nonequilibrium models assumed (Yakimowski and Barrett 2014b). Although the present study has made progress in understanding the mechanisms by which subdioecy originates in *S. latifolia*, the adaptive basis and long-term fate of this complex sexual system remains an open question.

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#### DATA ARCHIVING

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Figure S1.** The relation between K and Delta K for Structure analyses of 42 eastern North American populations of *Sagittaria latifolia*.

**Figure S2.** Hybrid index (q) as estimated by Structure for individuals in (A) monoecious populations, (B) dioecious populations, and (C) mixed populations of *Sagittaria latifolia*.