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Clonal genetic structure and diversity in populations of an aquatic plant with combined vs. separate sexes

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Abstract

Clonality is often implicated in models of the evolution of dioecy, but few studies have explicitly compared clonal structure between plant sexual systems, or between the sexes in dioecious populations. Here, we exploit the occurrence of monoecy and dioecy in clonal *Sagittaria latifola* (Alismataceae) to evaluate two main hypotheses: (i) clone sizes are smaller in monoecious than dioecious populations, because of constraints imposed on clone size by costs associated with geitonogamy; (ii) in dioecious populations, male clones are larger and flower more often than female clones because of sex-differential reproductive costs. Differences in clone size and flowering could result in discordance between ramet- and genet-based sex ratios. We used spatially explicit sampling to address these hypotheses in 10 monoecious and 11 dioecious populations of *S. latifola* at the northern range limit in Eastern North America. In contrast to our predictions, monoecious clones were significantly larger than dioecious clones, probably due to their higher rates of vegetative growth and corm production, and in dioecious populations, there was no difference in clone size between females and males; ramet- and genet-based sex ratios were therefore highly correlated. Genotypic diversity declined with latitude for both sexual systems, but monoecious populations exhibited lower genotypic richness. Differences in life history between the sexual systems of *S. latifola* appear to be the most important determinants of clonal structure and diversity.

Keywords: clonality, dioecy, monoecy, population structure, range limits, *Sagittaria latifolia*

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Introduction

Perennial plants often combine sexual reproduction with some form of asexual reproduction (Fryxell 1957; de Kroon & van Groenendael 1997). Clonal reproduction allows genotypes to expand spatially through vegetative production of shoots (hereafter ramets, Harper 1977), and clonal propagules facilitate dispersal (Vallejo-Marín *et al.* 2010). There are several explanations for the prevalence of clonality among perennial plants including the ability of clones to acquire and share resources among ramets, the greater chance of establishment and survival of clonal propagules relative to seeds, a reduced likelihood of genet death with increasing numbers of ramets and the twofold fitness advantage relative to sexual reproduction (Grace 1993; de Kroon & van Groenendael 1997; Silvertown 2008; Vallejo-Marín *et al.* 2010). Angiosperm clonal strategies complicate the sampling of genetic variation and genet identification. Molecular markers provide the most reliable means for characterizing the genetic structure of clonal populations (Parks & Werth 1993; Reusch *et al.* 2000; Alberto *et al.* 2005).

Clonal growth can also exert constraints on sexual reproduction with both ecological and evolutionary consequences (reviewed in Vallejo-Marín *et al.* 2010). For example, resource allocation trade-offs between vegetative growth and sexual reproduction may reduce ramet and genet reproductive expenditure (Van Drunen & Dorken 2012). Second, clonal growth can interfere with

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outcrossing in hermaphroditic populations, owing to increased opportunities for pollen transfer between flowers of a clone (geitonogamy). The number of flowering ramets increases as clones grow, and this intensifies the probability of this mode of self-fertilization. Geitonogamy has two principle mating costs: inbreeding depression and pollen discounting (Harder & Barrett 1995; Reusch 2001; Charpentier 2002). Indeed, increased selfing has been detected in association with increased clonality (Handel 1985; Reusch 2001; Albert *et al.* 2008; Somme *et al.* 2014). This leads to the prediction that clone size may differ between hermaphrodite vs. dioecious populations, because in the latter, any constraints on clonal expansion imposed by the costs of geitonogamy are relieved owing to unisexuality. However, the relation between clonality and sexual-system variation has not been investigated explicitly in flowering plants.

Despite the theoretical prediction that 1:1 sex ratios should be maintained by negative frequency-dependent selection, plant sex ratios exhibit wide variation (Barrett *et al.* 2010; Sinclair *et al.* 2011; Field *et al.* 2013a). However, the majority of sex ratio estimates are based on flowering individuals because of the inability to identify the gender of nonreproductive plants. Moreover, because dioecy in angiosperms is often associated with clonal propagation (Field *et al.* 2013a,b), data on sex ratios are largely based on flowering ramets, raising the question of whether ramet sex ratios accurately reflect genet sex ratios in clonal populations. The typically higher cost of reproduction associated with fruit and seed maturation may limit allocation of resources to clonal growth in females, resulting in smaller clone sizes and/or reduced flowering compared to males (Lloyd & Webb 1977; Delph 1999; Barrett & Hough 2013). If this occurs, there may be a weak relation between ramet and genet sex ratios within populations.

Genotypic diversity in clonal populations provides an indirect estimate of the relative success of sexual vs. asexual reproduction (Ellstrand & Roose 1987; Silvertown 2008). Populations in which most recruits result from sexual reproduction should contain higher diversity than populations in which clonal propagation dominates. Also, how genetic diversity is spatially structured depends on dispersal distances of pollen, seeds and clonal propagules, as well as the type of sexual system and clonal strategy of a species (e.g. 'guerilla' vs. 'phalanx'; see Charpentier 2002; Vekemans & Hardy 2004; Vallejo-Marín *et al.* 2010). Spatial patterns of isolation-by-distance and the degree of relatedness among ramets and genets can inform inferences about the relative importance of sexual vs. clonal reproduction in affecting spatial genetic structure. Estimates of spatial structure based on ramets involve the combined effects of both sexual and clonal reproduction, whereas the effect of clonality can be removed by investigating the spatial structure of genets (Reusch *et al.* 1999; Alberto *et al.* 2005). Fine-scale mapping and the use of genetic markers are required to conduct these types of analyses.

Variation in sexual and asexual reproduction among populations may also influence broad-scale geographic patterns of genetic diversity in clonal plants. In some species, sexual reproduction diminishes at range limits owing to a variety of biotic (e.g. lack of pollinators) and abiotic (cool temperatures and shorter growing season) factors (Eckert 2002). This can be accompanied by a decline in sexual fertility and a reduction in genetic diversity at range limits (Eckert & Barrett 1993; Dorken *et al.* 2004). If sexual reproduction is restricted in clonal plant populations at range margins, we might predict larger clone sizes in comparison with populations in less marginal habitats, as more resources may be available for clonal growth.

Sagittaria latifolia (Alismataceae), a widespread clonal aquatic, is useful for evaluating the scenarios discussed above. Populations are most commonly either monoecious (hermaphroditic plants) or dioecious (unisexual plants) enabling comparisons of clone size, genotypic diversity and spatial genetic structure. Populations can also be subdioecious, in which females and males co-occur with significant numbers of hermaphrodite plants (Yakimowski & Barrett 2014); however, in this study, we focused on dioecious populations, with only three containing a low frequency (<10%) of hermaphrodites. *Sagittaria latifolia* is also suitable for examining the relation between ramet and genet sex ratios because dioecious populations exhibit a wide range of variation in flowering ramet sex ratios (see Fig. 2 in Yakimowski & Barrett 2014).

We mapped and sampled leaves from vegetative and flowering ramets in 10 monoecious and 11 dioecious populations of *S. latifolia* in Ontario and Quebec, where the northern range limit of dioecy occurs. We sampled populations during peak flowering when it is straightforward to distinguish populations of the two sexual systems. Using genetic markers to identify clones, we addressed the following questions: (i) Are ramet and genet sex ratios correlated among dioecious populations? (ii) Are male clones larger than female clones and do they flower more frequently? (iii) Are clones larger in dioecious than monoecious populations, as predicted if unisexuality relieves constraints on clone size imposed by geitonogamy? (iv) Do monoecious populations exhibit stronger spatial genetic structure than dioecious populations, as might occur because of their opportunities for selfing? (v) Does clonal genetic diversity decrease with latitude, particularly in dioecious populations as they approach their northern range limit?

Methods

Study system

Sagittaria latifolia occurs in wetland habitats throughout Eastern North America with the northern range limit of dioecy occurring ~46°N and monoecy extending to ~52°N (Bogin 1955). Clones of *S. latifolia* are composed of vegetative and flowering ramets with inflorescences pollinated by generalist insects, including bees, flies and wasps (Glaettli & Barrett 2008). In dioecious populations, the gender of reproductive ramets is easily determined from buds, flowers or by the presence of fruit. Clonal propagation occurs through two mechanisms: (i) stolon formation and the growth of daughter ramets and (ii) corm production at the terminus of stolons at the end of the season. In Ontario and Quebec, ramets emerge in early June and flowering begins in July (monoecious populations) and August (dioecious populations) continuing until plants senesce in September, with populations regenerating from corms and seed the following spring. Earlier studies indicate that in this region, the two sexual systems of *S. latifolia* are ecologically differentiated with contrasting life history traits (Dorken *et al.* 2002; Dorken & Barrett 2003). Briefly, ramets are smaller in monoecious populations and produce many corms, whereas ramets in dioecious populations are larger and produce fewer, larger corms. These differences are associated with contrasting habitat requirements; monoecious populations are more frequent in open, ephemeral habitats, whereas dioecious populations most commonly occur in stable wetlands dominated by *Typha* stands.

Sampling strategy and genotyping

We sampled 10 monoecious and 11 dioecious populations of *S. latifolia* in Ontario and Quebec, evenly distributed from southern Ontario to the northern range limit of dioecy at ~46°N (Fig. 1). Populations were sampled during peak flowering from July–August 2009 and 2010. We used a spatially explicit sampling design to allow for comparisons of clonal structure and diversity among populations and sexual system. Details of our fine- and coarse-scale quadrat sampling protocol are in Fig S1 (Supporting information). For both types of sampling, we recorded the sex phenotype for all flowering ramets and collected leaf tissue.

For all 52 fine-scale quadrats within each monoecious and dioecious population, we assayed genetic variation for one focal ramet, or the closest ramet if a focal ramet was absent. To estimate genet sex ratios and the relative size of female and male clones, we increased our sample size by mapping locations of all flowering ramets across fine- and coarse-scale sampling areas and chose 1–12 ramets (mean = 3.0) for genotyping from each patch of ramets clustered together of the same sex phenotype. If there were patches > 2 m^2 without flowering ramets in the coarse-scale sample, we haphazardly selected 1–11 (mean = 5.2) representative ramets from these areas for genotyping, enabling assignment of non-flowering ramets to clones that were flowering in other parts of the population. In addition to the 52 fine-scale ramets sampled, an average of 54.3 (range 11–96) additional ramets were genotyped in dioecious populations. We extracted DNA and amplified 11 polymorphic microsatellite loci for each sample following methods in Yakimowski *et al.* (2009). PCR products were genotyped by The Centre for Applied Genomics (TCAG) in Toronto, ON, Canada.

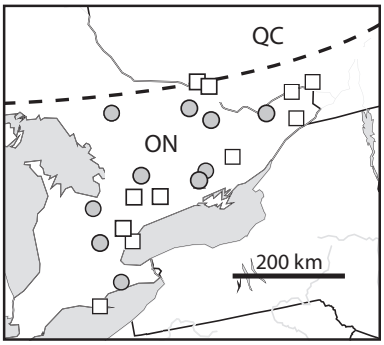

Fig. 1 The geographical distribution of the 10 monoecious (grey circles) and 11 dioecious populations (white squares) of *Sagittaria latifolia* sampled in this study. The northern range limit of dioecious populations is indicated by the dotted line.

Due to varying quality of leaf tissue, we were not able to assay SSR variation for all individuals and loci. Therefore, for each population, we deleted loci and/or individuals with missing data to optimize the number of individuals and loci retained in a data set with no missing data. On average, we deleted 3.4 of 11 loci and 8.5 of 52 samples; sample sizes and the number of loci used for each population are in Table S1 (Supporting information).

Clone assignment

For each population, ramets were assigned to genets using the 'Multilocus Match' function in GENALEX 6.2 (Peakall & Smouse 2006). To determine the probability of overestimating clonality (P_{sex}) due to lack of marker polymorphism, we first calculated the probability of a multilocus genotype occurring (P_{gen}) based on l, the number of loci, h, the number of heterozygous loci,

and allele frequencies f and g. To obtain a more conservative estimate of P_{sex}, we used an estimate of P_{gen}, the probability of each multilocus genotype, which accounts for deviations from Hardy–Weinberg equilibrium, such as those that occur due to selfing, by incorporating an estimate of F_{IS} (GENCLONE 2.0: Parks & Werth 1993; Arnaud-Haond & Belkhir 2007; Arnaud-Haond *et al.* 2007).

$$P_{gen}(F_{IS}) = \prod_{i=1}^{l} [(f_i g_i) \times (1 + (z_i \times (F_{IS_{(i)}})))] 2^h$$

Next, we calculated the probability that a subsample of n ramets sharing the same multilocus genotype arose via sexual reproduction given the total sample of N ramets, P_{sex} (GENCLONE 2.0: Parks & Werth 1993; Arnaud-Haond & Belkhir 2007; Arnaud-Haond *et al.* 2007):

$$P_{sex} = \sum_{i=n}^{N} \frac{N!}{i!(N-i)!} (P_{gen})^i (1 - P_{gen})^{N-i}$$

Therefore, as marker diversity increases (low P_{gen}) and the number of observations of the same multilocus genotype increases (n), the probability that ramets resulted from clonal reproduction becomes higher (low P_{sex}). In four monoecious populations, P_{sex} was estimated to be >0.05 for at least one genet, suggesting that some identical multilocus genotypes among these genets may have arisen by sexual reproduction. We corrected for this by multiplying the number of ramets assigned to these multilocus genotypes by the probability that all ramets with the same multilocus genotype arose via clonal growth ($1–P_{sex}$). In two of the four monoecious populations, this decreased the number of ramets assigned to the genet by at least one ramet for two genets per population. These four corrected values were used in our estimates of clone size (see below).

We corrected for potential overestimates in the number of genets in a population arising from scoring errors or somatic mutations; these can cause an overrepresentation of genotypes with very small differences in genetic distance. We used GENODIVE v. 2.0b2 (Meirmans & Van Tienderen 2004) to identify the threshold genetic distance above which scoring errors and mutations are unlikely to affect assignment of ramets to genets (Arnaud-Haond *et al.* 2007). For each population, we examined the distribution of genetic distances between individuals and manually set the threshold distance above any visible peak in the distribution of genetic distances (see Fig. S2, Supporting information). GENODIVE then assigns genotypes differing by genetic distances below the threshold to the same clonal lineage. Seven populations were unaffected by this correction, and overall it decreased estimates of R by 0.09 (range = 0–0.43). All subsequent analyses that involve estimates of clone size were performed with both clonal assignments from raw data and this corrected data. Results from the raw data are presented below, and the corrected data corroborate these results unless otherwise noted. Finally, we used a Monte Carlo resampling procedure (GENCLONE 2.0; 'resample loci' with 1000 replicates) to determine whether the number of SSR loci used for each population provided sufficient power to distinguish all multilocus genotypes in the sample.

Comparisons within dioecious populations: sex ratio estimates and clone size

For each dioecious population, we estimated the frequency of female and male flowering ramets (ramet sex ratio). We estimated the genet sex ratio by assigning sex phenotypes to clones with at least one flowering ramet. The mean proportion of clones for which sex phenotype could be identified was 57% (range 43–95%), and on average, the gender of 34 clones (range 9–61) per population was determined. We performed Pearson product-moment correlations to test the similarity between ramet and genet estimates of population sex ratios.

We calculated the per cent of ramets sampled that were represented by each multilocus genotype (hereafter 'clone size'). To investigate differences in flowering propensity of female and male clones, we compared the proportion of ramets flowering between female and male clones using generalized mixed models with a binomial distribution (R 2.8.1, package lme4), with sexual system as a fixed effect and population as a random effect. The significance of the population factor was tested by likelihood ratio tests with one degree of freedom. Using GENALEX v. 6.2 (Peakall & Smouse 2006), we estimated clone area (m^2), the maximum area (m^2) covered by ramets of the same multilocus genotype, from the raw multilocus genotype data and the spatial location of ramets (x–y coordinates). We compared mean area between female and male clones with a linear mixed model with clone sex as a fixed effect and population as a random effect.

Comparison between monoecious and dioecious populations

Clonal diversity. For each monoecious and dioecious population, we measured alleles per locus and genotypic diversity. The mean number of alleles per locus was $\log_{10}$-transformed and compared between monoecious and dioecious populations with a linear model. Given the number of multilocus genotypes (G) and individual ramets (N), we estimated genotypic richness for each population as:

$$R = \frac{G-1}{N-1},$$

which varies from 0 to 1, where 1 represents all ramets with a unique multilocus genotype (Dorken & Eckert 2001).

We analysed the effect of latitude and sexual system on genotypic richness (R) using a generalized linear model with a binomial distribution (using R v. 2.8.1). Explanatory variables included sexual system (monoecious or dioecious), latitude (continuous) and the sexual system x latitude interaction. We compared clone size and clone area between sexual systems using analogous statistical models. Clone area was $\log_{10}$ transformed, and variation among monoecious and dioecious populations examined using a linear mixed model (library 'nlme', R v. 2.8.1) fit by restricted maximum likelihood with sexual system as a fixed effect and population as a random effect.

Spatial architecture. For each population, we performed an analysis of spatial autocorrelation of multilocus genotypes with a matrix of spatial distances calculated from the sampling grid of each population. For each population, we performed a ramet- and genet-level analysis using GENCLONE 2.0. (Arnaud-Haond & Belkhir 2007), which examines the relation between relatedness and spatial distance for all pairs of ramets and pairs of ramets from different genets, respectively. Pairwise spatial distance values were binned into 10 categories; the average number of pairs per bin for the ramet-level analysis was 94 (range = 40–113) and 34 (range = 9–95) for the genet-level analysis. We estimated relatedness (F_{ij}) among pairs of ramets (i and j) using two methods:

(1) Hereafter referred to as 'Loiselle' method (Loiselle *et al.* 1995):

$$\hat{F}_{ij} = \sum l \frac{\left[\sum a(p_{ila} - p_{la})(p_{jla} - p_{la}) + \sum a\left(p_{la}\left(\frac{1-p_{la}}{n_l-1}\right)\right)\right]}{\Sigma l \, \Sigma a(p_{la}(1-p_{la}))}$$

where p_a is the frequency of allele a at locus l, and n_1 is the number of alleles at locus l

(2) Hereafter referred to as 'Ritland' method (Lynch & Ritland 1999; Arnaud-Haond *et al.* 2007):

$$\hat{F}_{ij} = \frac{\Sigma l(\Sigma a \, \Sigma ci \, \Sigma cj \, ((x_{lcia} x_{lcja}/p_{la})/(\Sigma ci \Sigma cj 1))) - 1}{\Sigma l(m_l - 1)}$$

where l = locus and a = alleles, ci and cj are homologous chromosomes, and m_l represents the number of alleles at locus l.

For the genet-level analyses, we employed a resampling approach which randomly chooses one possible spatial coordinate for each multilocus genotype at each resampling iteration (Alberto *et al.* 2005). For each of the ramet- and genet-level analyses in each population, the magnitude of spatial autocorrelation is estimated by beta, the slope of the regression between spatial distance and F_{ij}. For the genet-level analysis, we calculated the P-value of the regression by randomly permuting spatial positions among genets, to simulate a random spatial distribution of multilocus genotypes, and then compared it to the observed distribution. For each population, we plotted the index of relatedness F_{ij} (Lynch & Ritland 1999) for each of the 10 distance intervals for both ramet and genet runs. The spatial scale at which the probability of encountering clone mates approaches zero (Harada *et al.* 1997; Arnaud-Haond *et al.* 2007) is referred to as 'clonal subrange' and corresponds to the distance interval on the spatial autocorrelogram where the ramet and genet curves converge (Alberto *et al.* 2005).

We also calculated Sp, an index of spatial genetic structure that unlike the two measures above makes no assumption regarding levels of heterozygosity and is thus useful for comparing populations that differ in mating system (Vekemans & Hardy 2004):

$$Sp = \frac{b_F}{(1 - F(1))}$$

We used the resulting beta and $F(1)$, the relatedness of individuals from the first and smallest distance interval, from the spatial autocorrelation analyses described above. Variation in clonal subrange and Sp was modelled with linear analysis of covariance with sexual system as a fixed effect and latitude as a continuous variable.

Results

Dioecious populations

Sex ratio estimates. Estimates of flowering ramet sex ratio among the 11 dioecious populations deviated from 1:1 in several populations; one population was significantly female biased and four were significantly male biased (Table S2, Supporting information). Sex ratio, estimated as female frequency, varied widely for both ramet (0.27–0.77) and genet estimates (0.30–0.61). However, mean ramet sex ratio (0.46) was very similar to genet sex ratio (0.44), and there was a strong correlation between ramet and genet estimates of sex ratio among populations (r = 0.89, d.f. = 10, P = 0.0002; Fig. 2).

Comparisons between female and male clones. There was no overall difference in clone size between female and male clones (GLMM: z-value = −1.43, P = 0.15). The

average female and male clone comprised 3.7% and 3.3% of the ramets sampled among all populations; note that these averages are small due to the large number of multilocus genotypes represented by a single ramet (Fig. 4). Excluding these single genotypes, on average, female and male clones made up 6.8 and 6.9% of the ramets sampled. Although populations differed in average clone size (LRT: $\chi^2 = 175.18$, d.f. = 1, $P < 0.0001$), there was no significant population × clone sex interaction ($\chi^2 = 0.00$, d.f. = 1, $P = 1$). Similarly, populations varied significantly in clone area (LRT: $\chi^2 = 8.69$, d.f. = 1, $P < 0.01$), but there was no difference in maximum area covered by female vs. male clones (LMM: $t = -0.36$, d.f. = 101, $P = 0.72$); the mean area of female and males clones was 1.08 and 1.39 m^2, respectively. Excluding unique genotypes, the mean area of female and male clones is 3.75 and 3.61 m^2, respectively.

Comparison of the proportion of ramets flowering within female and male clones revealed little to no difference between the sexes. Using raw clone assignments, there was a significant effect of clone sex (GLMM: z-value = 2.39, $P = 0.02$), owing to a higher mean frequency of flowering in male clones (0.87) compared to female clones (0.79). However, following correction for scoring errors/mutations, this difference in flowering was reduced (female clones = 0.72, male clones = 0.76) and not statistically significant (GLMM: z-value = 0.26, $P = 0.14$). Populations differed in the proportion of flowering ramets per clone ($\chi^2 = 90.514$, d.f. = 1, $P < 0.0001$), but there was no significant population × clone size interaction ($\chi^2 = 0.00$, d.f. = 1, $P = 1$).

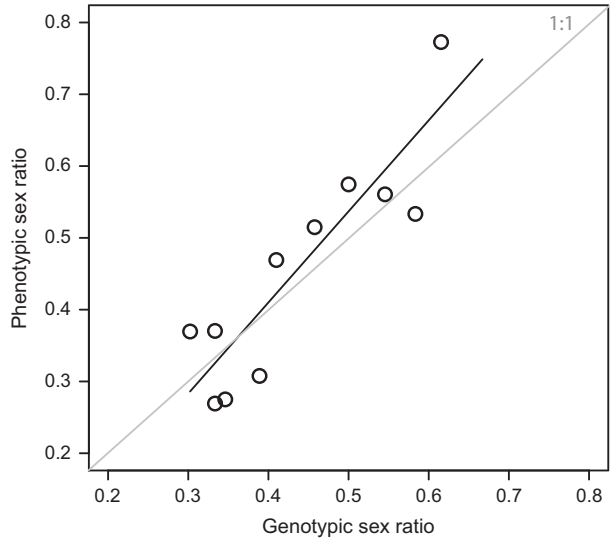

Fig. 2 The relation between ramet and genet sex ratio estimated as female frequency. Line of best fit based on linear regression is shown in black, and the 1:1 line is shown in gray.

Comparisons between monoecious and dioecious populations

Clonal diversity. Monoecious populations contained lower allelic diversity (mean alleles per locus = 3.98 ± 0.32) than dioecious populations (mean alleles per locus = 6.59 ± 0.33) (LM: $F_{1,19} = 8.3$, $P = 0.01$). Although the number of loci assayed in each population varied (range = 5–10; mean = 7.6), there was no significant difference between the average number of loci used in monoecious (mean 8.1 loci) vs. dioecious (mean = 7.2 loci) populations (LM: $F_{1,19} = 1.97$; $P = 0.18$). On average, the minimum number of loci needed to accurately determine genotypic richness was ~4, and in all populations, the number of loci used exceeded this value (Table S1, Supporting information). Genotypic richness (R) was significantly lower in monoecious populations (mean = 0.42; range = 0.30–0.66) than dioecious populations (mean = 0.63; range = 0.38–0.89 GLMM: z-value = −2.467, $P = 0.01$; Figure 5.4.A), despite higher ramet density in monoecious populations (see below). Overall genotypic diversity declined with latitude for both sexual systems (GLMM: z-value = −4.11, $P < 0.0001$). Using the raw clone assignments, this decline was significantly steeper for dioecious than monoecious populations (GLMM: sexual system x latitude: z-value = 2.33, $P = 0.02$) (Fig. 3c); however, using the scoring error/mutation correction, the interaction was not significant and the model with interaction was not the best-fit model (Appendix S1, Supporting information).

The density of all ramets within populations of *S. latifolia* (see Fig. S1, Supporting information) was significantly higher (GLM: z-value: 11.64, $P \leq 0.0001$) in monoecious (mean = 0.46; range = 0.21–0.75) than dioecious populations (mean = 0.40; range = 0.25–0.69). Latitude was not a significant factor (GLM: z-value = −1.20, $P = 0.23$) in explaining patterns of ramet density; however, there was a significant sexual system by latitude interaction (GLM: z-value = −11.52, $P < 0.0001$). This was due to a decline in ramet density in monoecious populations, whereas dioecious populations exhibited wide variation throughout the range that was sampled.

Clone size. The frequency distribution of clone size, as measured by the per cent ramets sampled per multilocus genotype, for all populations was highly positively skewed due to a high proportion of multilocus genotypes represented by a single ramet (Fig. 4) [mean frequency of unique genotypes: monoecy 0.28 (range = 0.15–0.38); dioecy 0.50 (range = 0.23–0.85)]. Overall, the probability of obtaining through random mating the same multilocus genotype n times for the

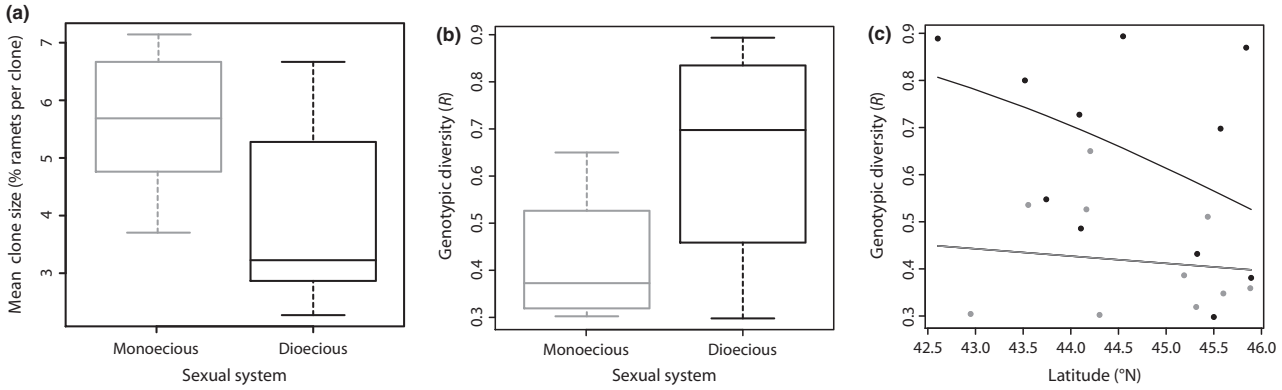

Fig. 3 Comparisons between monoecious (grey) and dioecious (black) populations of *Sagittaria latifolia*: (a) mean clone size estimated as the per cent of ramets sampled that were represented by each multilocus genotype corrected for P_{sex}, (b) genotypic diversity (R), and (c) the relation between latitude and R. In (a) and (b), the lower and upper limits of each box contain the middle 50% of the data, the horizontal line in each box is the median and whiskers extend to the minimum and maximum R values. In (c), the lines of best fit were derived from logistic regression model (black line = dioecious populations; grey line = monoecious populations).

sample sizes we used (N) was very low (mean P_{sex} = 0.03, population mean range = 6.7 × 10^{-10}–0.22); these probabilities were lower in dioecious populations (mean P_{sex} = 1.43 × 10^{-10}) than monoecious populations (mean P_{sex} = 0.05). After correcting the number of ramets per clone for the four clones with a P_{sex} value high enough to influence values of ramets per clone, we found that average clone size was significantly larger in monoecious (mean = 5.4%) than dioecious (mean = 3.7%) populations (GLMM: z-value = −2.81, P = 0.005). However, the spatial area of clones was not significantly different among populations (LRT: χ^2 = 1.51, d.f. = 1, P = 0.25) or between sexual systems (LMM: t = 1.76, P = 0.09). Including latitude did not

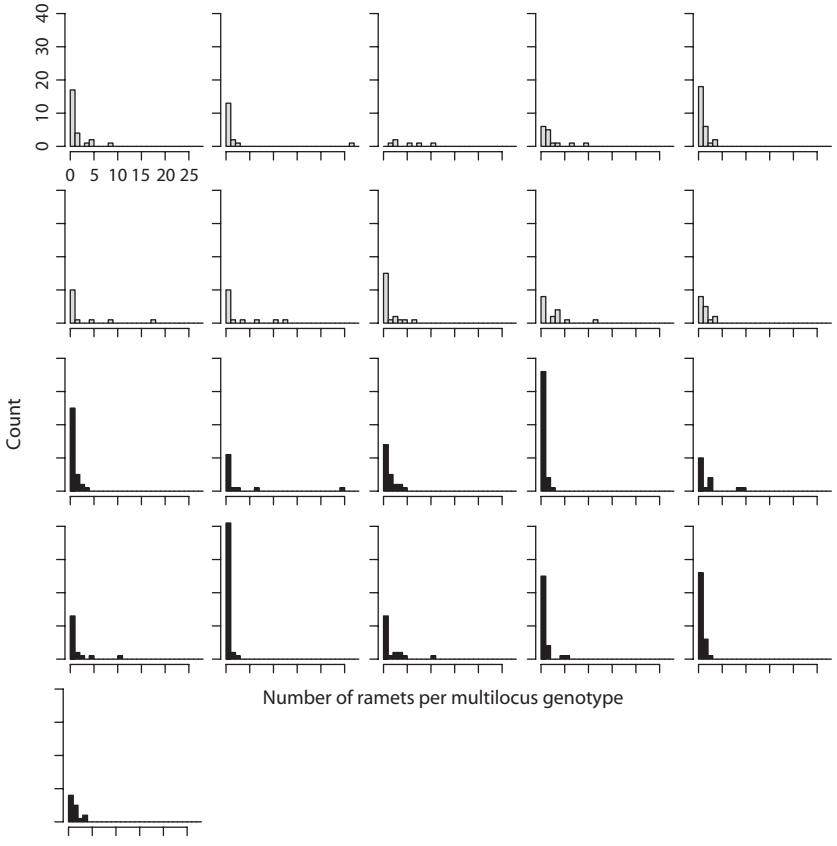

Fig. 4 Distribution of number of ramets associated with each multilocus genotype for 10 monoecious (gray bars) and 11 dioecious (black) populations of *Sagittaria latifolia*. Populations appear in the same order as Table S4 (Supporting information; regression analysis between these distributions and a power-law Pareto distribution) from top-left to bottom-right.

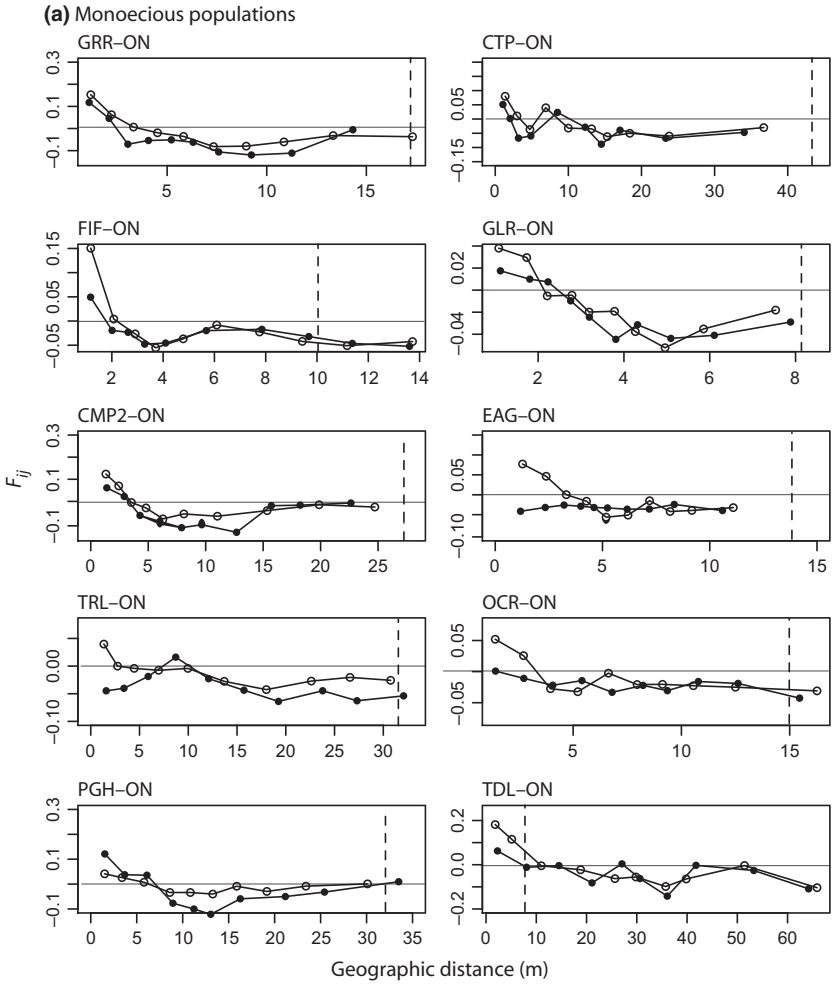

Fig. 5 Relation between relatedness (Ritland's F_{ij}) and geographic distance for (a) monoecious populations (circle symbols) and (b) dioecious populations (square symbols) of *Sagittaria latifolia* for the ramet (grey line)- and genet-level (black line) spatial autocorrelations. The vertical dotted line indicates the clonal subrange for each population. Regression statistics are reported for each population in Table 1.

improve the fit of the model and was not a significant factor in explaining variation in clone size.

Spatial architecture. Clonal subrange, an estimate of the spatial scale of clonality, was significantly higher in monoecious than dioecious populations (LM: $F_{1,19} = 19.01$, $P = 0.0003$; Fig. 5a, b). In monoecious populations, clones influenced genetic structure on spatial scales ranging from 7.4 to 43.0 m (mean = 20.5 m), whereas in dioecious populations, these values ranged from 2.2 to 8.0 m (mean = 6.7 m). Variation in latitude did not improve the fit of the model. We also examined variation in *Sp*, an index of spatial genetic structure for which higher values indicated greater spatial structure. At the ramet level, *Sp* estimates range from 0.0008 to 0.02 ($\text{mean}_{\text{Loiselle}} = 0.008$; $\text{mean}_{\text{Ritland}} = 0.006$) in monoecious populations and 0.001 to 0.06 in dioecious populations ($\text{mean}_{\text{Loiselle}} = 0.016$; $\text{mean}_{\text{Ritland}} = 0.012$). At the genet level, *Sp* estimates range from −0.001 to 0.01 ($\text{mean}_{\text{Loiselle}} = 0.004$; $\text{mean}_{\text{Ritland}} = 0.003$) in monoecious populations and −0.0001 to 0.04 in dioecious populations ($\text{mean}_{\text{Loiselle}} = 0.010$; $\text{mean}_{\text{Ritland}} = 0.007$). There was no significant difference between the two sexual systems for indices of spatial structure at the ramet (Sp_{Loiselle}: $F_{1,17} = 1.19$, $P = 0.29$; Sp_{Ritland}: $F_{1,17} = 1.37$, $P = 0.26$) or genet level (Sp_{Loiselle}: $F_{1,17} = 2.63$, $P = 0.12$; Sp_{Ritland}: $F_{1,17} = 1.88$, $P = 0.19$). And, although inclusion of latitude improved model fit, it was not found to be a significant explanatory variable for either index at ramet (Sp_{Loiselle}: $F_{1,18} = 0.97$, $P = 0.34$; Sp_{Ritland}: $F_{1,17} = 0.84$, $P = 0.37$) or genet levels (Sp_{Loiselle}: $F_{1,17} = 0.00$, $P = 0.97$; Sp_{Ritland}: $F_{1,17} = 0.00$, $P = 0.94$).

There were significant patterns of spatial autocorrelation of multilocus genotypes using relatedness coefficients at the ramet level for all but one monoecious (PGH-ON) and one dioecious (PTI-ON) population. This result was consistent using both the Loiselle and Ritland estimates of relatedness. The average slope and significance for the ramet-level regressions of Loiselle-relatedness and spatial distance (*m*) across monoecious and dioecious populations were $b = -0.007$, $P = 0.009$ and $b = -0.01$, $P = 0.02$, respectively. Similarly, the

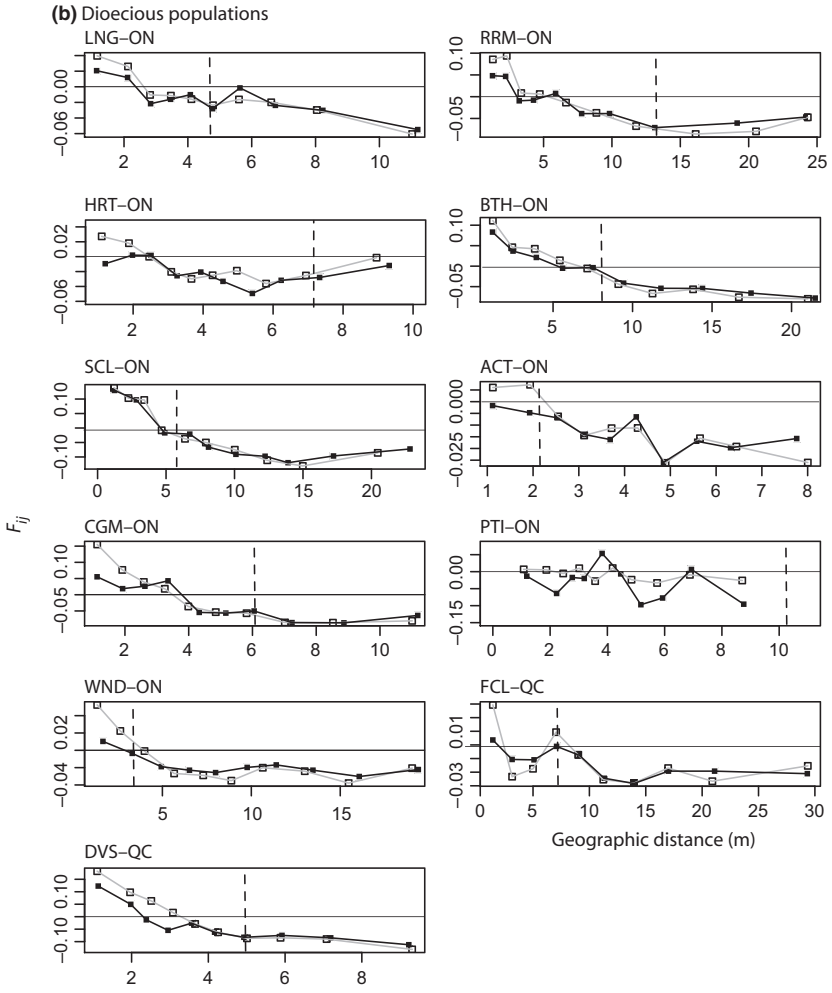

Fig. 5 *Continued*

average slope and significance for the regressions of Ritland-relatedness and spatial distance (m) across monoecious and dioecious populations were $b = -0.005$, $P = 0.007$ and $b = -0.01$, $P = 0.007$, respectively. However, at the genet level, the two estimates of relatedness differed; using the Loiselle estimator, there were significant patterns of spatial autocorrelation for 60% and 73% of monoecious and dioecious populations, respectively (Table 1). In contrast, the Ritland estimator gave substantially higher values with 70% of monoecious populations and 91% of dioecious populations with significant spatial autocorrelation.

For the genet-level analyses, the average slope and significance for the regression of Loiselle-relatedness and spatial distance (m) across monoecious populations were $b = -0.003$ and $P = 0.09$, respectively, and across dioecious populations $b = -0.008$ and $P = 0.04$. The average slope and significance for the regression of Ritland-relatedness and spatial distance (m) across monoecious populations were $b = -0.003$ and $P = 0.07$, respectively, and across dioecious populations $b = -0.007$ and $P = 0.01$. The higher rate of detection of spatial autocorrelation using the Ritland estimator is consistent with Vekemans and Hardy's (2004) finding that the Ritland estimator provides more statistical power to detect spatial autocorrelation when using hypervariable markers, such as the SSR markers used in our study.

Discussion

This study provides the first explicit comparison of clonal structure and diversity among plant populations differing in sexual system. It revealed the following major findings: (i) Clones in monoecious populations were significantly larger than those in dioecious populations; (ii) In dioecious populations, female and male clones were not significantly different in size and thus there was a close correlation between ramet and genet sex ratios; (iii) Among dioecious populations, ramet and genet sex ratios were highly correlated (Fig. 2); (iv) Genotypic diversity and allelic richness were significantly higher in dioecious than monoecious populations; (v) Genotypic

Table 1 Summary statistics for spatial genetic structure for monoecious and dioecious populations of *Sagittaria latifolia*. The slope (β) and *P*-values for the relation between relatedness (F_{ij}) and spatial distance (m) within each population were calculated at the ramet (left-hand columns) and genet level (right-hand columns). The index of spatial genetic structure (Sp) is derived from spatial autocorrelation parameters. All analyses were performed using both Loiselle and Ritland estimates of F_{ij}

	Ramet-level analysis						Genet-level analysis					
	Spatial autocorrelation				Index of spatial genetic structure		Spatial autocorrelation				Index of spatial genetic structure	
Population	$\beta_{Loiselle}$	$P_{Loiselle}$	$\beta_{Ritland}$	$P_{Ritland}$	$Sp_{Loiselle}$	$Sp_{Ritland}$	$\beta_{Loiselle}$	$P_{Loiselle}$	$\beta_{Ritland}$	$P_{Ritland}$	$Sp_{Loiselle}$	$Sp_{Ritland}$
Monoecious												
GRR-ON	−0.011	0	−0.0086	0	0.015	0.010	−0.011	0.001	−0.0092	0	0.014	0.010
CTP-ON	−0.0024	0.02	−0.0024	0.003	0.0028	0.0027	−0.0013	0.05	−0.0019	0.01	0.0014	0.0020
FIF-ON	−0.0063	0.001	−0.0079	0	0.0076	0.0093	−0.0024	0.05	−0.0038	0.005	0.0026	0.0040
GLR-ON	−0.017	0.002	−0.010	0.002	0.018	0.011	−0.014	0.004	−0.0086	0.004	0.015	0.0087
CMP2-ON	−0.0036	0.01	−0.0033	0.006	0.0043	0.0038	−0.00062	0.3	−0.00077	0.3	0.00069	0.00082
EAG-ON	−0.017	0	−0.010	0	0.020	0.011	0.0012	0.4	−0.00010	0.3	−0.0012	0.00010
TRL-ON	−0.0033	0	−0.0016	0	0.0037	0.0016	−0.0023	0.003	−0.0012	0.003	0.0024	0.0012
OCR-ON	−0.0042	0.001	−0.0040	0	0.0045	0.0042	−0.0029	0.05	−0.001	0.06	0.0030	0.0019
PGH-ON	−0.00074	0.06	−0.0011	0.06	0.00077	0.0012	−0.0020	0.06	−0.0022	0.05	0.0023	0.0025
TDL-ON	−0.0041	0	−0.0033	0	0.0055	0.0041	−0.0011	0.09	−0.0017	0.02	0.0012	0.0018
Dioecious												
LNG-ON	−0.011	0	−0.0084	0	0.012	0.0088	−0.0075	0	−0.0060	0	0.0077	0.0062
RRM-ON	−0.014	0	−0.0063	0	0.017	0.0069	−0.0082	0	−0.0042	0	0.0093	0.0044
HRT-ON	−0.0035	0.04	−0.0043	0.005	0.0037	0.0044	−0.0018	0.13	−0.0027	0.07	0.0018	0.0027
BTH-ON	−0.015	0	−0.0089	0	0.019	0.010	−0.012	0	−0.0071	0	0.014	0.0078
SCL-ON	−0.016	0	−0.013	0	0.02	0.015	−0.011	0	−0.0096	0	0.013	0.011
ACT-ON	−0.0061	0.003	−0.0047	0	0.0063	0.0047	−0.0018	0.1	−0.0026	0.003	0.0018	0.0025
CGM-ON	−0.024	0	−0.022	0	0.029	0.026	−0.016	0	−0.014	0	0.018	0.015
PTI-ON	−0.0024	0.10	−0.0045	0.06	0.0024	0.0045	−0.0067	0.05	−0.0078	0.04	0.0068	−0.00013
WND-ON	−0.0053	0	−0.0033	0	0.0059	0.0035	−0.0016	0.02	−0.0014	0.003	0.0016	0.0015
FCL-QC	−0.0013	0.03	−0.00096	0.009	0.0014	0.00099	−0.0010	0.07	−0.00079	0.03	0.0011	0.00078
DVS-QC	−0.047	0	−0.036	0	0.064	0.044	−0.029	0	−0.023	0	0.036	0.027

diversity declined with increasing latitude. Below we interpret these findings in the context of the contrasting life histories and reproductive systems of populations, taking into account their ecological and geographical circumstances.

Sex ratios, clone size and flowering in dioecious populations

There was a close correspondence (~90%) between ramet and genet sex ratios. This is probably because dioecious populations were genotypically diverse rather than dominated by a few large clones, minimizing the potential for biases in ramet sex ratio. Moreover, we found that the flowering propensity of male clones was on average only slightly higher than female clones. Therefore, differences in the extent of clonal growth between sexes, and/or differences in flowering propensity are unlikely to contribute significantly to the large observed range of sex ratio variation in the species.

Despite evidence in *S. latifolia* for trade-offs between allocation to female function and ramet and corm production (Van Drunen & Dorken 2012), female and male clones were not significantly different in size (based on the proportion of total ramets per genotype), or in the area they covered. A similar result was reported in *Rumex acetosella* (Fujitaka & Sakai 2007) in which clone size did not differ between the sexes. The lack of increased allocation to clonal spread in males relative to females may be because female clones compensate for their higher cost of reproduction. For example, females may produce more ramets than males early in the season, acquiring additional resources later in the season when fruiting occurs (Delph 1999). A recent study of *S. latifolia* (Van Drunen & Dorken 2012) manipulated investment in female and male reproduction and found the expected evidence of a higher cost to female function − plants that matured seed exhibited reduced vegetative growth and produced fewer corms of lower mass. However, female corms exhibited higher nitrogen stores than male corms, perhaps resulting in greater competitive ability early in the growing season and counterbalancing the lower number and smaller size of female corms compared to males. Studies of the rate of growth and competitive ability of ramets from female vs. male corms are needed to understand whether females compensate for higher costs of reproduction.

Clonal characteristics in monoecious vs. dioecious populations

In contrast to earlier predictions that dioecious clones may be larger than monoecious clones in *S. latifolia*, due to a release from the constraints of geitonogamy (Barrett *et al*. 2001; Dorken & Barrett 2003), our results indicate the opposite pattern. This is probably because monoecious populations are capable of greater rates of clonal proliferation than dioecious populations. Both common garden and field comparisons have demonstrated that monoecious populations produce twice the number of ramets and corms than dioecious populations during the growing season (Dorken & Barrett 2003). Because monoecious populations often occur in ephemeral environments, we favour the hypothesis that there is stronger selection for mechanisms of numerical increase than in dioecious populations, where competitive interactions probably play a more important role (Dorken & Barrett 2003). Our results suggest that life history differences between sexual systems in *S. latifolia* influence relative clone size more than any constraints that might be imposed by geitonogamy.

Our study of microsatellite loci revealed that dioecious populations on average contain significantly more genotypic diversity and allelic richness than monoecious populations. An earlier comparison of *S. latifolia* using 12 allozyme loci found no significant difference in several measures of genetic variation between populations of the two sexual systems (Dorken *et al.* 2002). However, the different results are not unexpected as the greater variability at microsatellite loci is likely to increase the power to detect contrasting patterns of genetic variation relative to allozymes (Eanes 1999; Leffler *et al*. 2012).

Several nonmutually exclusive hypotheses may explain why there is more genetic diversity in dioecious than monoecious populations. Differences in population size between dioecious and monoecious populations may play a role. Ramet census data from 51 dioecious and 34 monoecious populations sampled from the same geographic area as this study demonstrated that dioecious populations were significantly larger in size (LM: $F_{1,83} = 10.9$, $P = 0.001$; data from Yakimowski & Barrett 2014). Also, the difference in genotypic diversity could arise because of differences in mating system: higher levels of diversity are expected in obligately outcrossing populations than in those with mixed mating (Hamrick & Godt 1989). A comparison of outcrossing rates between dioecious and monoecious populations of *S. latifolia* indicated the expected high levels of outcrossing in dioecious populations, and considerable selfing in monoecious populations (mean $s = 0.41$, $n = 6$ populations; Dorken *et al.* 2002). Our analysis of selfing rates (Table S3, Supporting information) confirmed that monoecious populations experience a range of selfing rates. The contrasting life history traits of dioecious and monoecious populations (Dorken & Barrett 2003) probably also play an important role in influencing levels of genetic diversity. Population census data indicated much more frequent local extirpation of monoecious

than dioecious populations (Dorken & Barrett 2003). Lower diversity in monoecious populations may therefore be associated with the more ephemeral nature of populations and greater likelihood of colonization by single founders, favoured by the hermaphrodite condition of monoecious plants.

Genotypic diversity in *S. latifolia* populations is structured geographically with an overall decline with latitude. Reduced genetic diversity at range limits is often found in plant populations (reviewed in Eckert *et al.* 2008). We have no evidence for declining population size or increase in isolation for dioecious range-limit populations (Yakimowski & Barrett 2014). In this study, ramet density declined with latitude for monoecious but not dioecious populations. However, we have documented a significant decline in the proportion of female flowers with latitude in dioecious populations, a feature highly correlated with seed production (Yakimowski & Barrett 2014), and it is possible that reduced reproductive capacity may contribute to lower seedling recruitment and genotypic diversity in northern populations. Seedling recruitment may also be reduced in northern populations due to a shorter growing season or unfavourable habitat conditions. Interestingly, there was no influence of latitude on clone size in dioecious populations despite the decline in genotypic diversity. This suggests that although seedling recruitment may be reduced in northern populations, clonality does not increase.

The spatial structure of *S. latifolia* clones also differed between monoecious and dioecious populations. This was manifested by different clonal subranges, the distance at which ramet- and genet-level estimates of spatial genetic structure converge (Arnaud-Haond *et al.* 2007). In monoecious populations, the clonal subrange was near or above the maximum spatial interval that we sampled. In contrast, in all dioecious populations (except HRT-ON), the clonal subrange occurred well within the spatial distances sampled (contrast Figs. 5a vs. b). Therefore, the pattern of isolation-by-distance in dioecious populations is not only generated by clonal reproduction but also by restricted pollen and seed dispersal. In monoecious populations, it was not possible to differentiate between the contributions of sexual and clonal processes to patterns of spatial autocorrelation because ramet- and genet-level patterns did not converge within the spatial scale examined.

The relatively weak pattern of spatial autocorrelation, even in the ramet-level analysis, reflects a moderate level of aggregation of clone mates and intermingling of ramets among clones (see Fig. S3, Supporting information). This is expected for a species in which the dispersal of clonal propagules occurs, and in which the intermingling of ramets is made possible by long stolons. *Sagittaria latifolia* is intermediate along the continuum of clonal strategies from the aggregated 'phalynx' strategy to the dispersed 'guerilla' strategy (Charpentier 2002; Vallejo-Marín *et al.* 2010). Low spatial genetic structure in *S. latifolia* could also arise from seedling recruitment, as seeds are small and readily dispersed in water. Indeed, there are several (the number depends on relatedness estimate) monoecious and at least one dioecious population for which we detected no significant pattern of spatial autocorrelation. Most of the average values of the index of spatial genetic structure (*Sp*) for *S. latifolia* were an order of magnitude lower than mean *Sp* values reported for trees (0.01) and herbs (0.04) by Vekemans and Hardy (2004), who did not explicitly consider the influence of clonality on *Sp*. The values that we estimated for monoecious (ramet: 0.007, genet: 0.002) and dioecious (ramet: 0.02, genet: 0.007) populations are comparable to the lowest values in their review. The generally low values of *Sp* we found are consistent with the overall finding that sexual reproduction plays an important role in shaping the population structure of *S. latifolia*, despite the species' prolific clonal reproduction.

Clone size and sexual-system evolution

Although it is not possible to determine historically what ecological conditions promoted the evolution of dioecy from monoecy in *S. latifola,* we can assess whether the parameters necessary for various selection hypotheses are plausible, given contemporary knowledge of the species' ecology and population genetics. The evolution of dioecy in *S. latifolia* has been proposed to have proceeded along the gynodioecious pathway (Dorken & Barrett 2003, 2004). According to these authors, females invade monoecious populations when clone sizes are sufficiently large that geitonogamous selfing results in strong inbreeding depression. Observed selfing rates and inbreeding depression measured in several *S. latifolia* populations meet the necessary requirements for female invasion (Dorken *et al.* 2002). This study has provided the first genetic estimates of clone size and has demonstrated that although clone sizes are quite variable, some monoecious populations contain large clones (e.g. PGH-ON, CMP2-ON, GRR-ON), and in such populations selfing rates may be large enough to promote the evolution of dioecy. We estimated variable amounts of selfing among the 11 monoecious populations based on the homozygosity of adult genets, but we detected no relation between clone size and selfing rate (LM: $F_{1,8} = 0.40$, $P = 0.54$). However, more precise estimates of selfing rate using seed families from assigned clones (Reusch 2001) are required to rigorously investigate the relation between clone size and selfing rate.

Without phylogenetic data on the correlated evolution of clone size and dioecy, it is difficult to determine whether large clone size preceded or followed the evolution of dioecy. This distinction in polarity is important because the former is required for the selection model described above, whereas the latter is expected according to the geitonogamy release hypothesis. Our results cast doubt on the second scenario, as clone sizes in dioecious populations were not significantly larger than in monoecious populations. Therefore, it seems quite probable that increased clone size, perhaps associated with habitat shifts, played an important role in the evolution of dioecy in *S. latifolia*.

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This work was part of the Ph.D. thesis of S.B.Y. in the Department of Ecology and Evolutionary Biology, University of Toronto and the work was supervised by S.C.H.B. S.B.Y. is interested in the evolution of plant sexual systems and ecology and evolution of populations at range margins; S.C.H.B. works on evolutionary transitions in plant reproductive systems and the genetics of colonization. S.B.Y. carried out the fieldwork and analyses and S.B.Y. and S.C.H.B. wrote the manuscript.

Data accessibility

The following listed data files necessary to replicate analyses presented in this manuscript, *R* code for relevant analyses, a ReadMe file documenting the content of data files, and which files are used for each analysis are available on Dryad doi:10.5061/dryad.gs7k6.

Dioecious populations

- Raw assignments of ramets to clones and sex phenotype: Clonality.Sagittaria.SexClones.xls
- Comparison of ramet (field collected data from flowering ramets) and genet sex ratio (based on SSR markers): Sexclone_summary.txt
- Comparison of size between female and male clones: CloneSize_FvsM.txt
- Comparison of flowering frequency in female and male clones after GenoDive correction: CloneSize_by-Clone_GenoDive_flwfreq.txt
- Genotype and spatial position data for each population formatted for GenAlEx: Clonality.Sagittaria.Sex-ClonesArea.xls
- Comparison of area between female and male clones: Clone Area_FvsM.txt
- Comparison of flowering frequency in female and male clones: Clone_Flw_Freq.txt
- Comparison of flowering frequency in female and male clones after GenoDive correction: CloneSize_by-Clone_GenoDive_flwfreq.txt

Monoecious and dioecious populations

- Raw assignments of ramets to clones: Clonality.Sagittaria.IDClones.xls

- Comparison of allelic diversity, genet diversity and clone size by sexual system: CloneSize_MvsD.txt
- Summary of Psex data and correction for relevant monoecious populations: Psex.correction.xls
- Comparison of clone area between monoecious and dioecious populations: CloneArea_MvsD.txt
- Comparison of ramet density and summary of spatial statistics by population: Spatial_MvsD.txt

Spatial analysis using GenClone

- input files for each population formatting for GenClone
- output files for each population showing results of spatial autocorrelation and clonal subrange. *Sp* calculations were added to each file after running software.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Populations of *S. latifolia* vary substantially in size and shape ranging from linear populations in drainage ditches to circular or quadrilateral shapes in shallow marshes.

Fig. S2 Choosing thresholds for clone assignment based on the distribution of genetic distance between individuals genotyped for 10 monoecious and 11 dioecious populations.

Fig. S3 Plots of the relative spatial positions of ramets on *X*–*Y* grid coordinates in: (a) monoecious population PGH-ON and (b) dioecious population PTI-ON.

Table S1 List of populations assayed for spatial genetic structure sorted by latitude.

Table S2 Summary of counts and frequencies of each sex phenotype (f = female, h = hermaphrodite, m = male) for both phenotypic and genotypic estimates of sex ratio in 11 dioecious populations of *S. latifolia*.

Table S3 Descriptive statistics of clonality, genetic diversity and ramet density for 10 monoecious and 11 dioecious populations of *Sagittaria latifolia*.

Table S4 Regression analysis of the relation between the frequency distribution of ramet number for each multilocus genotype (Fig. 4), and a power-law Pareto distribution for 10 monoecious and 11 dioecious populations of *Sagittaria latifolia*.

Appendix S1 Analysis of genotypic diversity in monoecious and dioecious populations using raw data clone assignments.