SEX RATIO VARIATION AND GENE FLOW AMONG POPULATIONS OF THE GYNODIOECIOUS *GERANIUM MACULATUM*

by

DOROTHY A. CHRISTOPHER

(Under the Direction of SHU-MEI CHANG)

ABSTRACT

Gynodioecy is a plant sexual system in which separate female and hermaphrodite individuals occur in a species. Population sex ratio is highly variable, which is unique among plant sexual systems with multiple sexes. We use *Geranium maculatum*, a gynodioecious species in which population female frequency varies from 0-50%, to investigate factors that may be important in determining the distribution of females. Non-selective forces such as gene flow and migration can be important in shaping the distribution of sexual phenotypes. Here we use a population genetic approach to identify patterns of genetic structure that may play a role in determining where females occur. We found decreased migration rates to and from populations with high female frequency. This suggests that frequency reintroduction of male sterility alleles is not the mechanism that maintains females in populations. We then investigated how fitness differences and patterns of sex allocation differ between the sexes and among populations that differ in their female frequency. Theory states that hermaphrodites should increase allocation to male function when females are present, however, we did not find evidence of this. Additionally, females did not have significantly higher seed production than hermaphrodites, however they do have larger rhizomes, which may allow females to increase lifetime fitness relative to

hermaphrodites. Finally, we considered pollen dispersal distances, and examined how these differ between populations. Pollen dispersal has implications for gene flow and the distribution of genetic variation in a population. We found that populations of high female frequency had reduced pollen dispersal distances. This pattern may perhaps be related to pollinator foraging patterns: previous work has shown pollinators discriminate against females in *Geranium maculatum*.

INDEX WORDS: gynodioecy, population genetics, pollen dispersal, gene flow, sexual system, Geranium

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CHAPTER 1

INTRODUCTION

While the overwhelming majority of flowering plant species produce hermaphroditic flowers, separate sexes have evolved independently numerous times across the angiosperm phylogeny (Renner 2014), suggesting there must be some selective benefit to separating sexual function among individuals (Richards 1997). Beginning with Darwin (1877), researchers have performed experimental and theoretical work to elucidate the benefits to unisexual flowers. These researchers have identified the avoidance of inbreeding depression due as an important advantage to separate sexes (Lloyd 1975; Charlesworth and Willis 2009).

After hermaphroditism, the most common sexual system is gynodioecy, in which females and hermaphrodites occur in a species. There is remarkable variation in the frequency of females in gynodioecious populations, with female frequencies varying between 0-90%, as in *Lobelia siphilitica* (Dudle et al. 2001), *Silene vulgaris* (Olson et al. 2005), and *Beta vulgaris* (Fénart et al. 2006). This is unique to gynodioecious systems, as we generally expect that when multiple sex morphs are present in a population, they should be at equal frequency due to negative frequency dependent selection. The sex ratio variation that is present across gynodioecious populations provides us with an opportunity to infer evolutionary histories between populations and investigate the role that the sex ratio plays in population dynamics.

Among population gene flow

Non-selective forces such as gene flow and migration can affect the patterns of sex ratio variation between populations. These forces can determine distribution of sex determination factors, which can affect the population female frequency and the likelihood that females can persist in populations. For example, work on metapopulation dynamics in *Silene vulgaris* has shown that population structure and gene flow within and among demes can maintain genetic variation (i.e., segregation at the locus that restores male function), as well as phenotypic variation (sex ratio variation among subpopulations) (Mccauley and Taylor 1997; Olson et al. 2005). Similarly, historical gene flow estimated by phylogeographic work on heterostylous species *Narcissus triandrous, Oxalis alpine,* and *Luculia pinceana* has revealed complex patterns of mating morph frequency among populations, and genetic drift that can cause the loss of morphs from some populations (e.g., Hodgins and Barrett 2007; Pérez-Alquicira et al. 2010; Zhou et al. 2012). In gynodioecious species, the role of migration and gene flow in determining patterns of sex ratio variation, specifically how females are introduced into populations, has not been fully investigated, which may limit our understanding of the complex dynamics that maintain gynodioecious systems.

Within population gene flow

Gene flow via pollen dispersal is important because it determines the patterns of mating and the distribution of alleles and the resulting genotypes and phenotypes in a population (Silvertown and Charlesworth 2001). Pollen dispersal distances, particularly in insect pollinated species, are generally expected to be limited, which creates challenges for reproduction in such species. With limited dispersal, even for a completely outcrossing species, the mixing of alleles

within a population may not be entirely panmictic, which can cause genetic structure within a population (Levin and Kerster 1974; Godoy and Jordano 2001).

Separate sexes can further affect patterns of pollen flow because the density of pollen-bearing individuals changes and, as a result, pollinator behavior may also change when visiting single sex plants (Fenster 1991). For example, the presence of females may influence pollinator behavior, as female flowers generally experience reduced pollinator visitation relative to hermaphrodites (reviewed in Delph 1996). This can shape patterns of pollen flow within a population in which females are present. In particular, restricted pollen flow can reduce the genetic neighborhood size for individuals in the population, which may cause individuals to experience a sex ratio that is not the population sex ratio, but rather the sex ratio of the local neighborhood. This in turn can affect the patterns of selection an individual experiences.

Selection on male and female traits

Gynodioecy is posited to be an intermediate stage in the evolution of dioecy from hermaphroditism. In the hermaphroditism to gynodioecy transition, females invade a population but can only be maintained if they can make up the fitness deficit caused by lack of pollen production. Hermaphrodites can gain fitness through pollen and seed production, but as females can only acquire fitness through seeds, they are at a fitness disadvantage. Thus females will establish in the population only if they can compensate for their lost siring ability. They must increase female function, which can be accomplished through having higher seed fitness, through either higher quantity or quality of seeds, than hermaphrodites (Charlesworth and Charlesworth 1978; Charlesworth and Ganders 1979).

The next stage is the evolution of dioecy. For dioecy to evolve, there are a set a theoretical predictions that must be met. Hermaphrodites are expected to become “more male” in their functional gender as female frequency increases: hermaphrodites will gain more of their fitness through male function. This is because the presence of females increases the number and availability of ovules in the population. This creates selective pressure that favors hermaphrodites with higher pollen production, and if this is coupled with a decrease in ovule and/or seed production, we would expect that hermaphrodites will become functionally and structurally more male (Spigler and Ashman 2012). There are few studies that have examined the gynodioecy-dioecy transition, particularly the dynamics of this shift in natural populations. Most of research that has been performed has utilized experimental arrays (McCauley and Brock 1998; Ehlers and Thompson 2004). However, we lack an understanding of how individuals allocate to male and female traits in natural populations.

Genetic control of male sterility

Identifying the genetic factors that control male sterility has long been a goal of researchers studying gynodioecious species; male sterility can be under either nuclear or cyto-nuclear control. Understanding the genetic control is important because it allows us to predict equilibrium population sex ratios as well as understand population dynamics such as the strength of selection on reproductive traits (Ashman and Majetic 2006; Barrett 2013). Conventional approaches have used reciprocal crosses between hermaphrodites, often with a known genetic background (i.e., whether it carries a male sterility allele and corresponding restorer of fertility). Progeny sex ratios can then be compared between individual crosses to understand the genetic

control and potentially the number of loci involved (e.g., Belhassen 1991; Dudle et al. 2001; Weller et al. 2014).

In *Geranium maculatum*, a previous crossing experiment has shown the genetic control of male sterility to be complex (Van Etten 2009). This study showed that there is most likely more than one nuclear locus involved. Van Etten found that when the distance between the parental populations of origin increased, the frequency of an intermediate phenotype increased, while the hermaphrodite frequency decreased. It is important to understand the factors that contribute to sex ratio variation to predict the long-term stability of gynodioecy as well as the population female frequency, which can influence selection on reproductive traits.

Dissertation research

Through my dissertation, I explored how within and among population gene flow shape the distribution of females, and how the sex ratio impacts the relative fitness of the two sexes. I also examined how population dynamics change based on the population sex ratio. In Chapter Two, I used a population genetic approach to assess gene flow patterns between twenty-eight populations that differed in their female frequency. In Chapter Three, I used the sex ratio variation found across ten populations of *Geranium maculatum* to examine how floral traits in hermaphrodites and females vary depending on the female frequency. I collected three years of field data to examine factors proposed to be important across different stages of the transition from hermaphroditism to dioecy pathway, including the female compensation necessary to maintain females and the increased allocation to male traits in hermaphrodites that precedes the evolution of dioecy. In Chapter Four, I quantified the distance that pollen travels in ten populations that range from 0-35% female. I examined how the presence of females and the

spatial structure of the sexes within a population influences pollen dispersal and the implications for intra-population gene flow. In Chapter Five, I performed a crossing experiment using a quantitative genetic approach to assess how the female frequency on the progeny arrays change based on the maternal and paternal populations of origin as well as the distance between parental populations.

Study system

Geranium maculatum L. (Geraniaceae) is a rhizomatous perennial herb found from Georgia north to southern Canada and west to the Great Plains (Bertin and Sholes 1993). It is commonly found is moist understory habitats, where it flowers from mid-April to mid-May. Seeds mature approximately one month later and are dispersed by elastic dehiscence of the schizocarp (Stamp and Lucas 1983). Hermaphrodite flowers produce two whorls of five anthers each. Individuals are self-compatible, however selfing rates in natural populations are quite low because flowers are strongly protandrous (Van Etten et al. 2014). Female flowers produce nonfunctional anthers that have been aborted at some point during development; anthers are small and do not produce pollen. Aboveground biomass including vegetative and inflorescence structures grow from one end of the rhizome. More than one growth point on a rhizome is rare in natural populations (personal observation).

References

Barrett, S. C. H. 2002. The evolution of plant sexual diversity. Nat. Rev. Genet. 3:274–84.
Bertin, R. I., and O. D. V Sholes. 1993. Weather, pollination and the phenology of Geranium maculatum. Am. Midl. Nat. 129:52–66.

Charlesworth, D., and B. Charlesworth. 1978. A model for the evolution of dioecy and gynodioecy. 112:975–997.

Charlesworth, D., and J. H. Willis. 2009. The genetics of inbreeding depression. Nat. Rev. Genet. 10:783–96.

Darwin, C. 1877. The different forms of flowers on plants of the same species. London.

Dudle, D. a., P. Mutikainen, and L. F. Delph. 2001. Genetics of sex determination in the gynodioecious species Lobelia siphilitica: Evidence from two populations. Heredity (Edinb). 86:265–276.

Etten, M. L. Van, A. C. Deen, J. L. Hamrick, and S. Chang. 2014. Mating system contributes only slightly to female maintenance in gynodioecious Geranium maculatum (Geraniaceae). 113:464–470.

Fénart, S., P. Touzet, J.-F. Arnaud, and J. Cuguen. 2006. Emergence of gynodioecy in wild beet (Beta vulgaris ssp. maritima L.): a genealogical approach using chloroplastic nucleotide sequences. Proc. Biol. Sci. 273:1391–8.

Garraud, C., B. Brachi, M. Dufay, P. Touzet, and J. a Shykoff. 2011. Genetic determination of male sterility in gynodioecious Silene nutans. Heredity (Edinb). 106:757–764.

Hodgins, K. a, and S. C. H. Barrett. 2007. Population structure and genetic diversity in tristylous Narcissus triandrus: insights from microsatellite and chloroplast DNA variation. Mol. Ecol. 16:2317–32.

Lloyd, D. G. 1975. The maintenance of gynodioecy and androdioecy in angiosperms. Genetica 45:325–339.

Lloyd, D. G. 1974. Theoretical sex ratios of dioecious and gynodioecious angiosperms. Heredity (Edinb). 32:11–34.

Mccauley, D. E., and D. R. Taylor. 1997. Local population structure and sex ratio: evolution in gynodioecious plants. Am. Nat. 150.

Olson, M. S., D. E. McCauley, and D. Taylor. 2005. Genetics and adaptation in structured populations: Sex ratio evolution in Silene vulgaris. Genetica 123:49–62.

Pérez-Alquicira, J., F. E. Molina-Freaner, D. Piñero, S. G. Weller, E. Martínez-Meyer, J. Rozas,

and C. a Domínguez. 2010. The role of historical factors and natural selection in the evolution of breeding systems of Oxalis alpina in the Sonoran desert “Sky Islands”. J. Evol. Biol. 23:2163–75.

Renner, S. S. 2014. The relative and absolute frequencies of angiosperm sexual systems: Dioecy, monoecy, gynodioecy, and an updated online database. Am. J. Bot. 101:1588–1596.

Richards, A. J. 1997. Plant Breeding Systems. 2nd ed. Garland Science.

van Damme, J. M. M., M. P. J. Hundscheid, S. Ivanovic, and H. P. Koelewijn. 2004. Multiple CMS-restorer gene polymorphism in gynodioecious Plantago coronopus. Heredity (Edinb). 93:175–81.

Weller, S. G., A. K. Sakai, T. M. Culley, L. Duong, and R. E. Danielson. 2014. Segregation of male-sterility alleles across a species boundary. J. Evol. Biol. 27:429–36.

Zhou, W., S. C. H. Barrett, H. Wang, and D. Z. Li. 2012. Loss of floral polymorphism in heterostylous luculia pinceana (rubiaceae): A molecular phylogeographic perspective. Mol. Ecol. 21:4631–4645.

CHAPTER TWO

POPULATION GENETIC STRUCTURE AND THE DISTRIBUTION OF A SEXUAL POLYMORPHISM IN THE GYNODIOECIOUS *GERANIUM MACULATUM*.[1]

[1]Christopher, Dorothy A. and S.-M. Chang. To be submitted to *Heredity*.

Abstract

Gynodioecy is a sexual system in which separate female and hermaphrodite sexes occur in a population. The patterns of selection on male and female traits have been studied extensively to identify the factors that promote female introduction and persistence in populations. Non-selective forces such as gene flow and migration have received much less attention, but have been shown in other systems to be important determinants of the distribution of sexual polymorphisms. Here we used a population genetic approach to examine the role of gene flow in introducing and maintaining females in *Geranium maculatum*. This species is common throughout the eastern US and females occur in a majority of the sampled populations. We found significant genetic structure between populations with high female frequency (HFF) (>0.25) and the rest of the sampled populations. Additionally, migration rates to and from high female frequency populations were reduced relative to populations with low female frequency or that were hermaphrodite-only. This suggests that frequent reintroduction of male sterility alleles does not maintain females in the HFF populations, as has been shown in other species. However, we cannot rule this out in the lower female frequency populations. Taken together, this highlights the complexity of female maintenance in this system.

Introduction

Plant sexual systems show remarkable diversity, ranging from hermaphroditism to separate male and female individuals. Separate sexes are thought to evolve largely to avoid the deleterious effects of inbreeding that can occur when hermaphrodites self (Lloyd 1974). Theoretical and empirical work has shown inbreeding depression to be an important selective pressure in the evolution of gynodioecy, in which separate female and hermaphrodite individuals are present in a species. Hermaphrodites can gain fitness through pollen and seeds, but females only produce seeds. They must therefore produce more or better quality progeny to coexist with hermaphrodites.

A unique characteristic of gynodioecious species is the highly variable population female frequency. In gynodioecious systems it is not unusual to find populations of some species with >75% female, while nearby populations are hermaphrodite-only (for example, *Lobelia siphilitica*, Case and Caruso 2007; *Silene vulgaris*, McCauley and Brock 1998; *Daphne laureola*, Alonso and Herrera 2011 and *Geranium maculatum*, *this study*). This phenomenon has been suggested to be the result of complex interactions between genetic and ecological factors (Dudle et al. 2001; Dufay et al. 2009; McCauley and Bailey 2009; Delph and Bailey 2010). Factors that promote female persistence have been studied extensively, with much of the work dedicated to determining the patterns of selection on traits that affect male and female fitness (e.g., Delph 1990; Mccauley and Brock 1998; Emery and McCauley 2002; Caruso and Yakobowski 2008; Hodgins and Barrett 2008; Dufay and Billard 2012). However, non-selective forces such as gene flow and migration can also affect the patterns of sex ratio variation between populations. Non-selective forces can determine distribution of sex determination factors, which can affect the population female frequency and the likelihood that females can persist in populations.

Despite their potential impact on the distribution of sexual polymorphisms, non-selective forces have received much less consideration than selection, yet their importance can be clearly seen across studies of different sexual systems. For example, work on metapopulation dynamics in *Silene vulgaris* has shown that population structure and gene flow within and among demes can maintain genetic variation (i.e., segregation at the locus that restores male function), as well as phenotypic variation (sex ratio variation among subpopulations) (Mccauley and Taylor 1997; Olson et al. 2005). Similarly, historical gene flow estimated by phylogeographic work on heterostylous species *Narcissus triandrous*, *Oxalis alpine*, and *Luculia pinceana* has revealed complex patterns of mating morph frequency among populations, and genetic drift that can cause the loss of morphs from some populations (e.g., Hodgins and Barrett 2007; Pérez-Alquicira et al. 2010; Zhou et al. 2012). Finally, knowledge of founder events and migration patterns, specifically the dispersal ability, has helped explaining the distribution of monoecious and dioecious populations of *Sagittaria latifolia (Dorken and Barrett, 2004).*

In gynodioecious species, the role of migration and gene flow in determining patterns of sex ratio variation, specifically how females are introduced into populations, has not bee fully investigated. Several possibilities exist for how females might be introduced into a population. First, male sterility mutations in natural populations may have arisen independently such that the presence of females in a population has little to do with migration events. In this scenario, we would not expect to see a relationship between the degree of genetic connectivity and the sex ratio. On the other hand, females in all populations may have been introduced by propagules originating from another population with male sterility alleles. In this case, we would expect to see an elevated migration rate between populations with females as well as higher genetic similarity between populations with females than between those with and without females.

Clearly, the mutation and migration events are not mutually exclusive and can work jointly in shaping the pattern of sex ratio variation among populations. However, population genetic approaches provide a powerful test for at least one of the scenarios – that migration is important in shaping the patterns of sex ratio variation that we observe.

Geranium maculatum is a gynodioecious perennial that is common throughout eastern North America. Females are present in a majority of the populations we have sampled (see Methods), which provides us with an opportunity to analyze patterns of genetic connectivity between populations that vary in their female frequency. To address how gene flow might affect the distribution of a sexual polymorphism, we used genetic markers to estimate gene flow and ask the following questions: 1) What is the role of gene flow and population structure in shaping the distribution of females across populations? 2) To what extent can neutral genetic variation explain the distribution of females?

Methods

Study System

Geranium maculatum L. is a rhizomatous perennial herb found from southern Canada south to the southeastern US and west to the Great Plains. Plants flower in mid-spring, producing 6-10 flowers per inflorescence (Agren and Willson 1991; Chang 2006). Flowers are generalist pollinated, mainly by small bees (Van Etten and Chang 2014). Hermaphrodites are self-compatible, although selfing rates in natural populations are fairly low (chapter 3; Van Etten and Chang 2014), likely because flowers are strongly protandrous. Fruits mature approximately one month after pollination, producing up to 5 seeds per fruit. Seeds are dispersed via dehiscence of the schizocarp; seeds are dispersed up to 3m from the maternal plant (Stamp and Lucas 1983).

Populations

In the spring of 2013, we located 28 populations across the eastern US (Fig. 2.1). Ten were hermaphrodite-only, while in 18 populations both sexes were present, with an average female frequency of 11% (Table 2.1). Though we did not exhaustively search for all populations in any areas during our collection, we did not discriminate between populations based on their sex ratio. Hence, our population information fairly reflects the natural pattern of female frequency distribution in the wild populations of this species. In each population, we collected leaf tissues from 24 individuals and preserved them in silica gel for later DNA extraction. In populations with females, we sampled from 12 females or all females if there were less than 12 in the population.

DNA extraction and genotyping

In each population we genotyped 24 individuals at 10 microsatellite loci. We extracted DNA using a modified CTAB protocol (Doyle and Doyle 1990). Microsatellite fragments were amplified using a 3-primer touchdown PCR program under the following conditions: 10 minutes of denaturing at 95 °C; followed by 10 cycles of denaturing at 95°C for 30 seconds, annealing at 65°C for 30 seconds, decreasing by 1°C every cycle, extension for 30 seconds at 72°C. This was followed by 30 cycles of denaturing at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension for 30 seconds at 72°C. This was followed by a final extension for 20 minutes at 72°C. PCR products were sized using an internal dye-labeled size standard (ROX500, UGA Georgia Genomics Facility) and analyzed on an ABI 3730 capillary sequencer. Allele calls were made using PeakScanner (ABI v1.0).

Data analyses

We calculated population genetic statistics using GenAlEx (Peakall and Smouse 2012) and FSTAT (Goudet 1995). We estimated percent polymorphic loci, heterozygosity, inbreeding coefficient, private alleles and tests of genetic differentiation (F_{ST}, G_{ST}). FreeNA (Chapuis and Estoup 2009) was used to identify null alleles by testing for heterozygote deficiency. We initially performed all analyses with females removed from the dataset to determine if females were genetically distinct from hermaphrodites, and thus any population genetic patterns may simply be due to the genetic make-up up females.

Mantel tests comparing geographic distance and $F_{ST}/(1-F_{ST})$ were used to assess isolation by distance in GenAlEx. Principal coordinate analyses (PCoA) were performed to visualize genetic structure between all populations using the *ade4* package in R v.3.2.3. Due to significant isolation by distance shown in the Mantel test and PCoA, we grouped populations by region for further analyses. To determine how to group populations into different regions, we performed an analysis of molecular variance (AMOVA) using GenAlEx, which partitions genetic variation hierarchically among regions and populations. We binned populations in different combinations, with an AMOVA performed on each. The grouping that yielded the highest among region variance explained was retained. Analyses were then performed separately on each region.

Within each region, we performed a PCoA to assess patterns of differentiation between populations. We evaluated historical migration using Migrate-n (Beerli and Palczewski 2010). Migrate-n uses a coalescent framework to determine pairwise migration rates between populations. To determine if migration patterns were due simply to geographic distance between populations, we analyzed the relationship between migration rate and distance using a linear model in R v.3.3.3 (R Core Development Team, 2017). We then examined the residuals from the

model—if migration rates were due to geography, we would expect that residuals from this model to be randomly distributed once the effect of geographic distance was removed. If the migration rates were independent of geography, we would expect to see the residuals correlated to the migration rates, because the effect of geography does not explain the observed migration rate values.

We also examined genetic structure using STRUCTURE within each region (Pritchard et al. 2000). STRUCTURE is a Bayesian clustering program that maximizes Hardy-Weinberg within clusters. Cluster membership coefficients were estimated using the correlated allele frequencies model and the *locprior* prior. We used 500,000 MCMC iterations after 100,000 burn in. We estimated the optimal number of clusters (K) using the delta K method described by Evanno et al. (2005) and calculated in Structure Harvester (Earl 2011).

Results

Genetic diversity

Analyses with females omitted were qualitatively very similar to the whole dataset (Table 2.5), therefore females were included in all analyses presented below. The 10 loci used in this study had an average of 8 alleles per locus. The majority of locus by population combinations showed no evidence of null alleles; 14 combinations had null alleles estimated to be at low frequency (<0.03). Null alleles were identified by testing for heterozygote deficiency; inbreeding can also cause heterozygote deficiency, however the selfing rate in natural populations of *G. maculatum* is very low (<0.012, Christopher, ch 3, 2017; Van Etten and Chang 2014), therefore this analysis is likely estimating true null alleles. Because null alleles were at low frequency, we included all 10 loci in further analyses.

Observed heterozygosity differed significantly from expected heterozygosity in 3 populations (Table 2.2). Allelic richness (R_s) was similar among all populations (Table 2.1). In neither of these measures did populations with and without females differ significantly ($F_{1,671}$=21.39, p=0.773). We did detect a difference in the number of private alleles: populations with females possessed more private alleles than hermaphrodite-only populations (mean 4.88 vs. 3).

Genetic structure

When analyzing all 28 populations, genetic structure is high (F_{ST} = 0.214, G_{ST} = 0.225). Populations exhibit a pattern in which populations that are geographically distant are also more genetically differentiated, which is consistent with isolation by distance (Fig. 2.2; R^2 = 0.600, p = 0.021). This is supported by a PCoA that includes the entire dataset, which shows populations separate along principal coordinate 1 (PC1 explains 41% of the variation), which follows a north-south gradient (Fig. 2.3).

To avoid the problem of conflating isolation by distance and genetic structure (Meirmans 2012) we partitioned populations into three regional groups, a southeast, central, and northeast region (Fig. 2.4). The designation of these regions is supported by an AMOVA; when populations were grouped in this way, as compared to other groupings, we detected the highest percent variance explained among region (Table 2.3).

PCoAs carried out for each region separately reveal an interesting pattern that is consistent across the three regions. In the southeast region, populations separate along the first PC axis, which explains 21% of the variation (Fig. 2.5a). The two populations with the highest female frequency, SR2 (32% female) and SU (34% female), separate from the rest of the populations in that region: SR2 and SU have values that are more negative on both the first and

second axis. In the central region, populations again separate along the first axis (26% of the variation), and the population with highest female frequency (BCSP, 28% female), falls out with more negative values on the first and second axis (Fig. 2.5b). Finally, in the northeast region, we detected a western subregion and an eastern subregion, which separate along the first principal component (20% of the variation explained). Within the western subregion, the two populations with the highest female frequency, LS (20% female) and TSF (18% female), again, separate from the rest of the populations in the region along the second axis, which explains 12% of the variation (Fig. 2.5c).

The STRUCTURE analysis revealed results consistent to the PCoA analyses. In the southeast, STRUCTURE identified K=3 clusters. Both high female frequency (HFF) populations had the highest proportion membership to the same cluster (Fig. 2.6a); these populations had more negative values on axis 1 of the PCoA (Fig. 2.5a). In the central region, STRUCTURE identified K=3 clusters again, with the HFF population (RBR) having the highest proportional assignment to a cluster not shared by the rest of the populations (Fig. 2.6b); this population had more negative values along axis 1 and axis 2 of the PCoA. In the northeast region, STRUCTURE identified K=4 clusters (Fig. 2.6c). Membership coefficients indicate the west and east subregions cluster separately, corresponding to more negative and positive values, respectively, along PC axis 1. The HFF populations form an additional cluster; these had more negative values on PC axis 2.

Migration rates to and from HFF populations were lower than any other estimates that did not involve HFF populations (ie, only involved hermaphrodite-only and low female frequency populations; Table 2.4). In the southeast, the lowest migration rates were from the two HFF populations - SU (32%) and SR2 (34%). In these two populations, the migration rate ranged

from 1.2-2.6. In hermaphrodite-only populations, the highest migration rate was 12.1, from R113 to R16, which are two hermaphrodite-only populations. In the central region, BCSP, a HFF population, had the lowest migration rates, ranging from 1.1-2.2. The highest migration rate was 9.8, from LR to PM, both of which are hermaphrodite-only populations. In the northeast, two HFF populations, LS and TSF, had the lowest migration rates, from 1.1-2.1. The differences in migration rates to and from HFF populations and the rest was significant (Wilcoxon signed rank test, $W=1030$, $p<-.001$).

There was a trend in which populations that were farther apart geographically had lower migration rates, which suggests some degree of isolation by distance within regions (Table 2.4). This relationship between migration rate and distance might suggest that the migration rates among all populations were due simply to geographic distance (and not due to female presence). We examined this by regressing the migration rate against the geographic distance for each region separately. We found distance explained a low to moderate level of the variation in migration rates (Fig. 2.7; southeast, $R^2=0.61$, $p<0.001$; central, $R^2= 0.65$, $p<0.001$; northeast, $R^2=0.29$, $p=0.08$).

Discussion

We first examined patterns of genetic diversity across the sampled populations. We found a clear pattern of significant isolation by distance across the sampled range, from Georgia north to New York. Isolation by distance is predicated on the null assumption that geographic distance is important in determining genetic differentiation among population such that geographically closer individuals will experience increased mating relative to individuals that are geographically distant. In *G. maculatum*, seeds are dispersed by the mechanic force of the schizocarp

dehiscence. Though this force can fling seeds to some distance away from the maternal plants, seeds rarely disperse more than a few meters from the maternal plants. Secondary seed dispersal is lacking in this species, making long distance gene flow via seeds likely rare events, resulting in isolation by distance. This is common among herbaceous understory species, and in fact most plant species show some amount of isolation by distance. This is mainly due to species' recolonization and expansion toward the poles after the last glacial maximum (Holliday et al. 2010).

The key finding of this study was that populations with high female frequency (HFF) are generally more genetically isolated from other populations. This is supported by lower migration rates to and from HFF populations, as well as PCoA analyses and STRUCTURE, which showed genetic structure between the HFF populations and the rest. Additionally, there were more private alleles in HFF populations, which supports reduced gene flow in these populations. The decreased migration rates were not only to due to geographic distance. These results taken together suggest that the HFF populations are genetically distinct from the others, and they are genetically isolated.

One of our objectives in this study was to examine how females are introduced to populations. In other species, females are introduced and maintained via frequent migration of male sterility alleles into a population, which maintains a pool of females in the population. In *Silene vulgaris*, metapopulation dynamics play a large part in sustaining females in populations. In this context, frequent reintroduction of cytoplasmic male sterility genes allows these genes to escape the corresponding nuclear restorer of male fertility, thus maintaining the male sterile phenotype among demes (reviewed in Olson et al. 2005). Additionally, structured demes may affect the fitness of male sterile genotypes relative to the case of no population structure.

However, in *Geranium maculatum*, metapopulation dynamics do not seem to be an important factor in the introduction and maintenance of females. If they were, we would expect to see high degrees of genetic connectivity between populations, and in particular we might expect to see high levels of gene flow into populations with females. Instead we saw the opposite pattern, and populations with high female frequency had decreased amounts of gene flow. This result suggests that there is genetic structure between HFF populations and the rest.

As introduced earlier, several possibilities exist for how females could be introduced and maintained in populations with low levels of gene flow. The low levels of genetic connectivity to and from HFF populations suggest that male sterility alleles may not be introduced via migration from other populations. Another explanation is that male sterility mutations arise independently in these populations, and thus migration of male sterility alleles into these populations is unnecessary. It may seem farfetched that a mutation that causes the same phenotype could arise independently in many populations; however, the presence of multiple male sterility loci in a species is not uncommon in gynodioecious species. For example, multiple loci within and among populations have been shown to occur in *Beta vulgaris* (Fénart et al. 2006; Dufay et al. 2009), *Silene vulgaris* (Sloan et al. 2012), and *Lobelia siphilitica* (Dudle et al. 2001). These loci are generally found in the mitochondrial genome. Since plant mitochondrial genomes often experience high levels of structural rearrangements and inversions (Hanson 1991; McCauley and Olson 2008; Sloan et al. 2012), it is possible that multiple mitchondrial mutations can result in similar male sterile phenotypes. In this situation, we would not expect to see a relationship between gene flow rates and the sex ratio, because the presence of male sterility alleles in a population is independent of migration; this is the pattern that we saw with regards to HFF populations.

However, not all populations with females were isolated: populations with low female frequency (between 1-12% female) did not show decreased migration rates. Therefore, we cannot rule out that male sterility alleles are introduced to a population via a migration event. The fact that we see variation in the migration rates of populations with females suggests that both gene flow and within-population dynamics play a role, underscoring the complexity of this system.

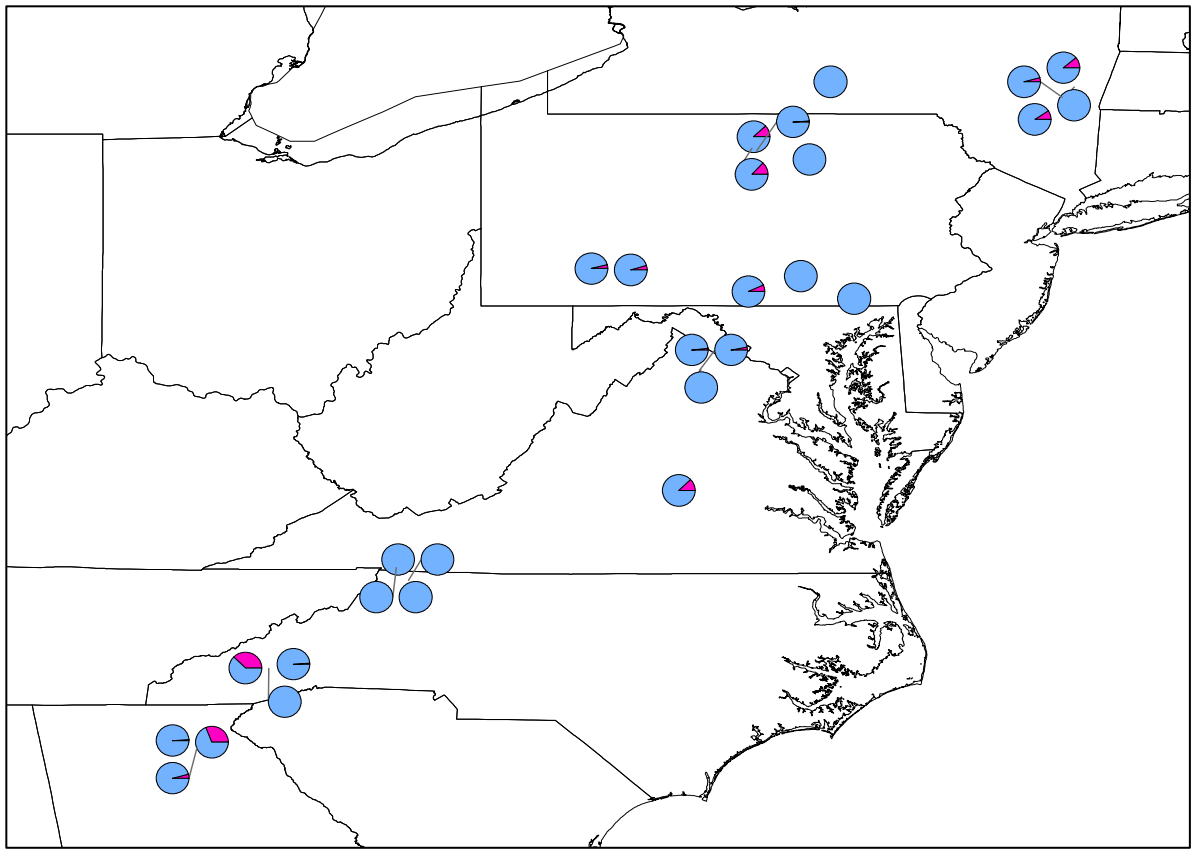


Fig. 2.1. Locations of sampled populations. Blue and pink indicate the proportion of the population hermaphrodite and female, respectively.

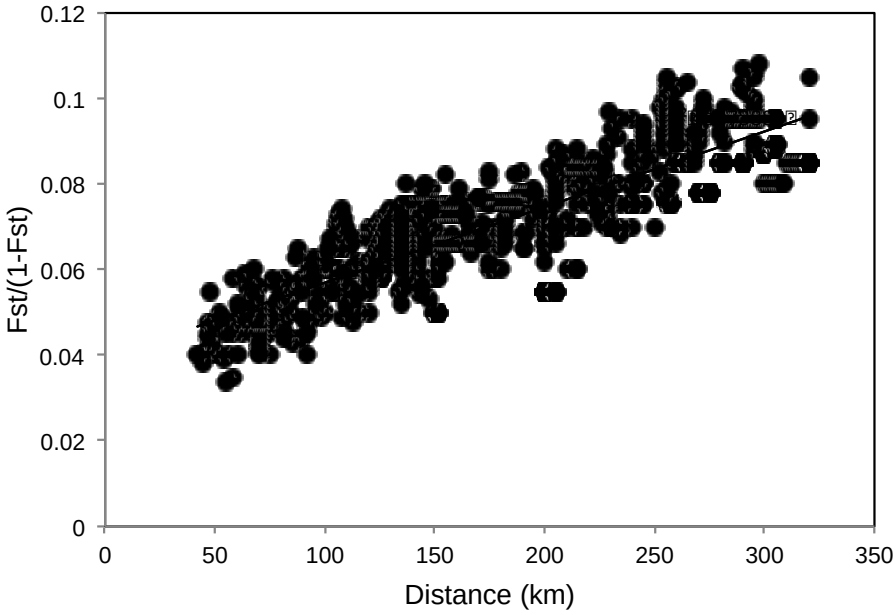


Fig. 2.2. Isolation by distance for sampled populations of *G. maculatum* ($R^2 = 0.73$, $p<0.01$).

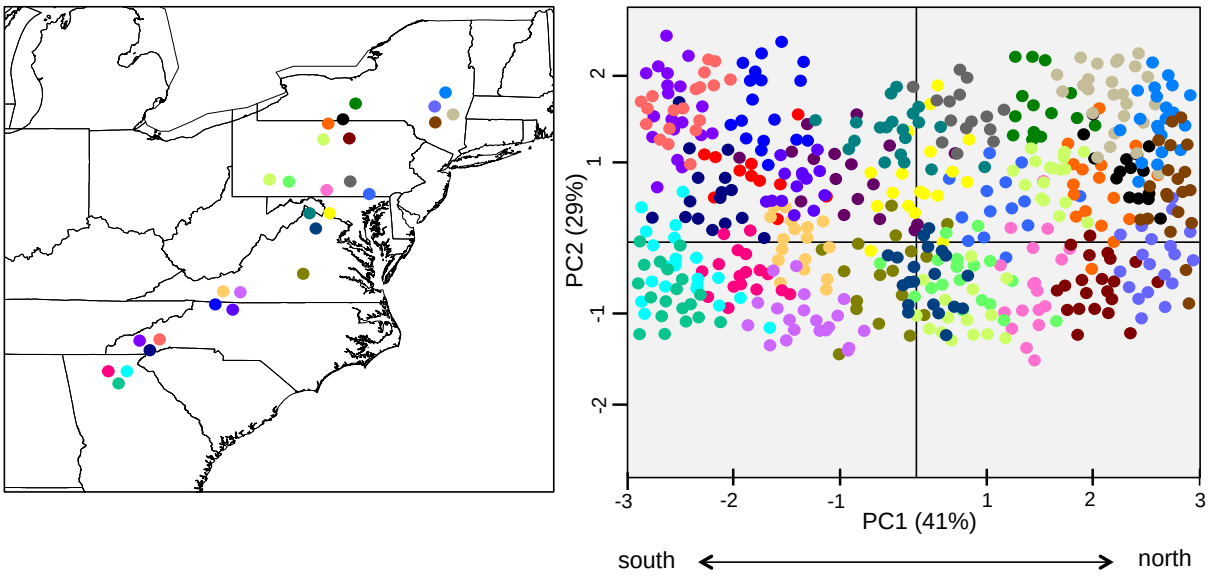


Fig. 2.3. Principal coordinates analysis of pairwise genetic distances of all 28 sampled populations. Colors correspond to the population of origin. Populations separate along axis 1, which corresponds to a north-south gradient.

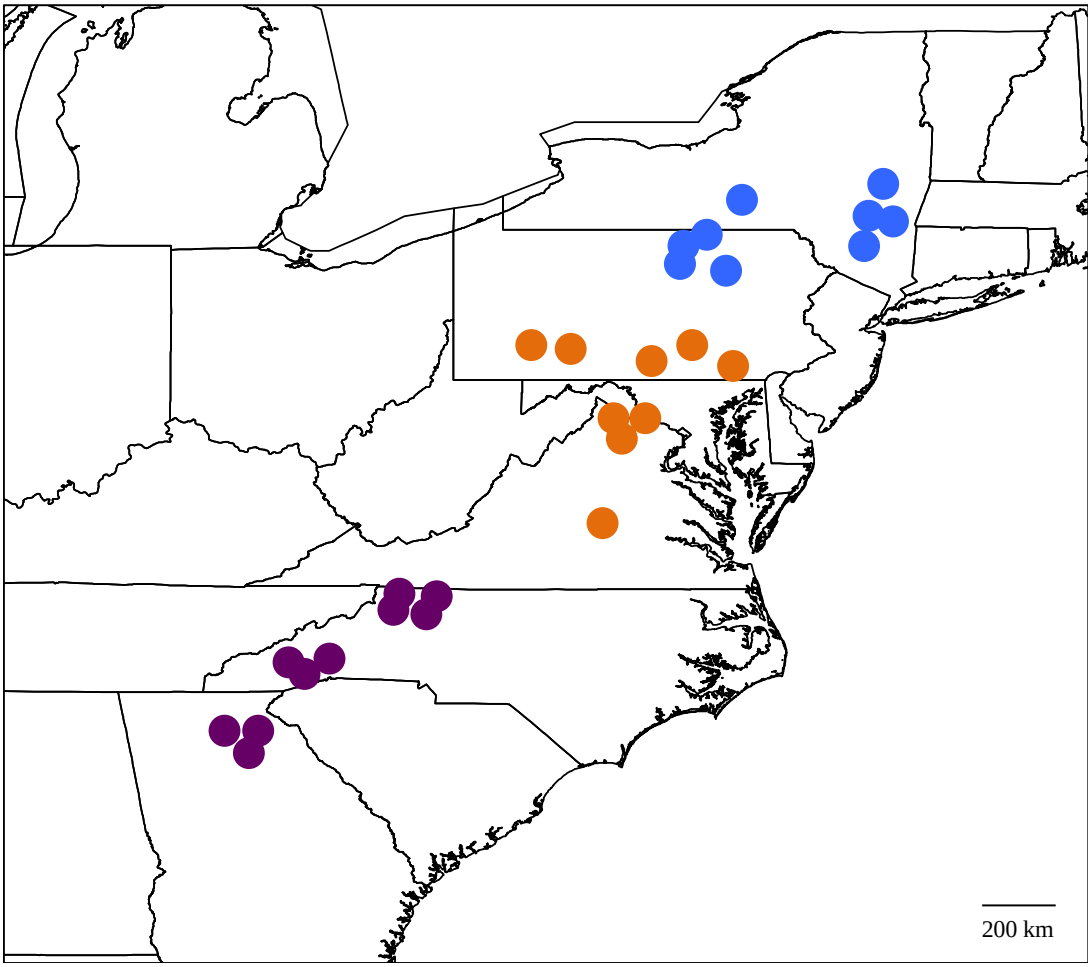


Fig. 2.4. Populations were divided into three regions for analyses, based on AMOVA results. Colors correspond to the region into which a population was placed: purple, southeast; orange, central; blue, northeast.

a)

b)

c)

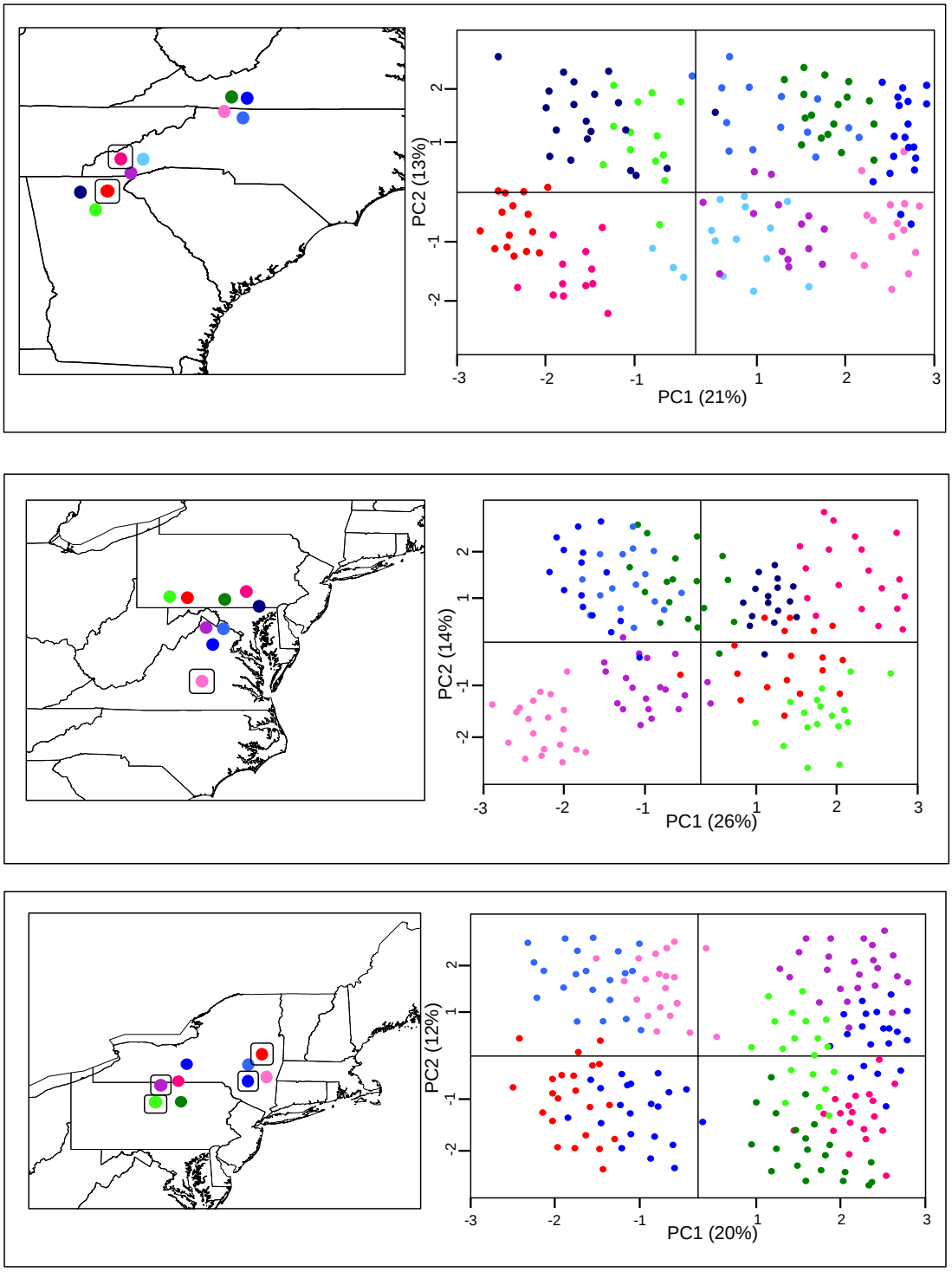


Fig. 2.5. Principal coordinates analyses of genetic distances among populations in the a) southeast, b) central, and c) northeast regions. Colors in PCoA plot correspond to colors of the population point on the map. High female frequency populations are circled on the map.

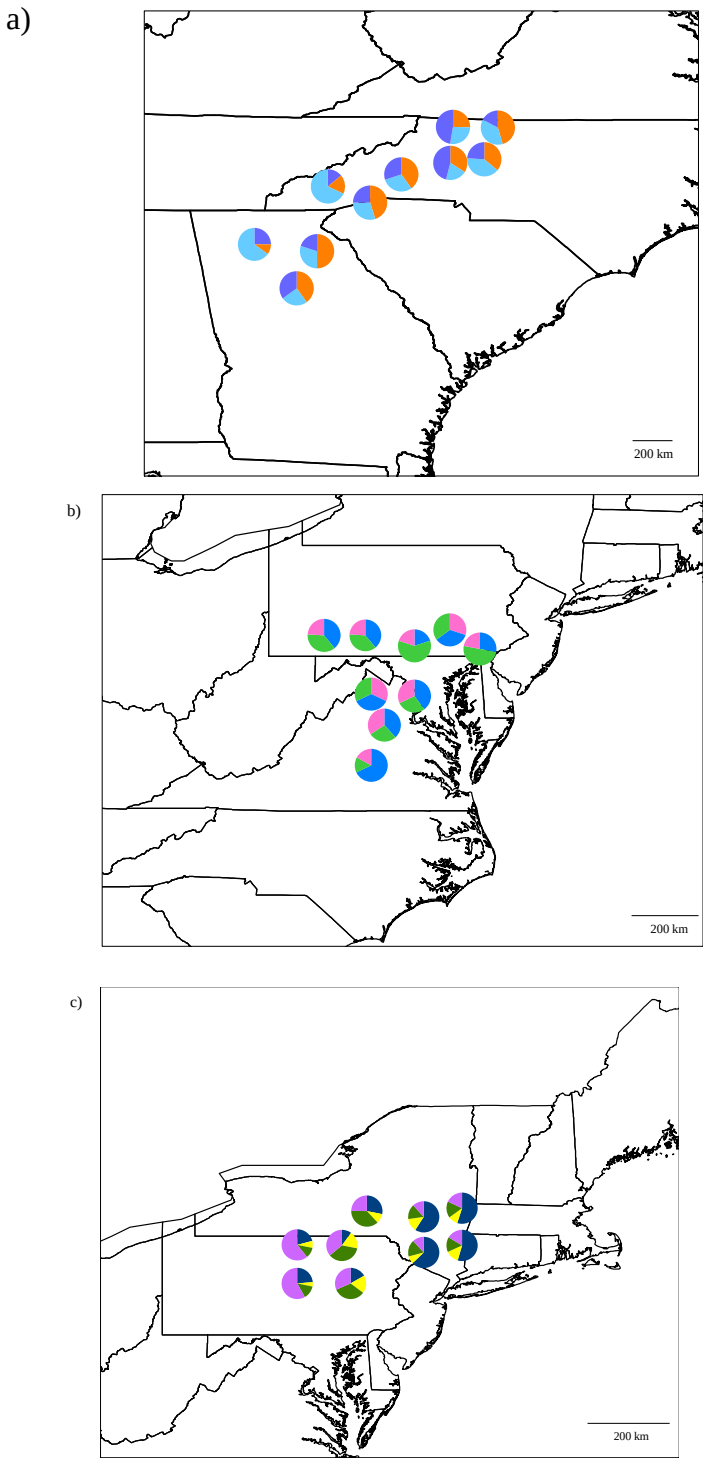


Fig. 2.6. STRUCTURE results for a) the southeast region (K=3), b) the central region (K=3), and c) the northeast region (K=4). Colors indicate proportional assignment to each cluster.

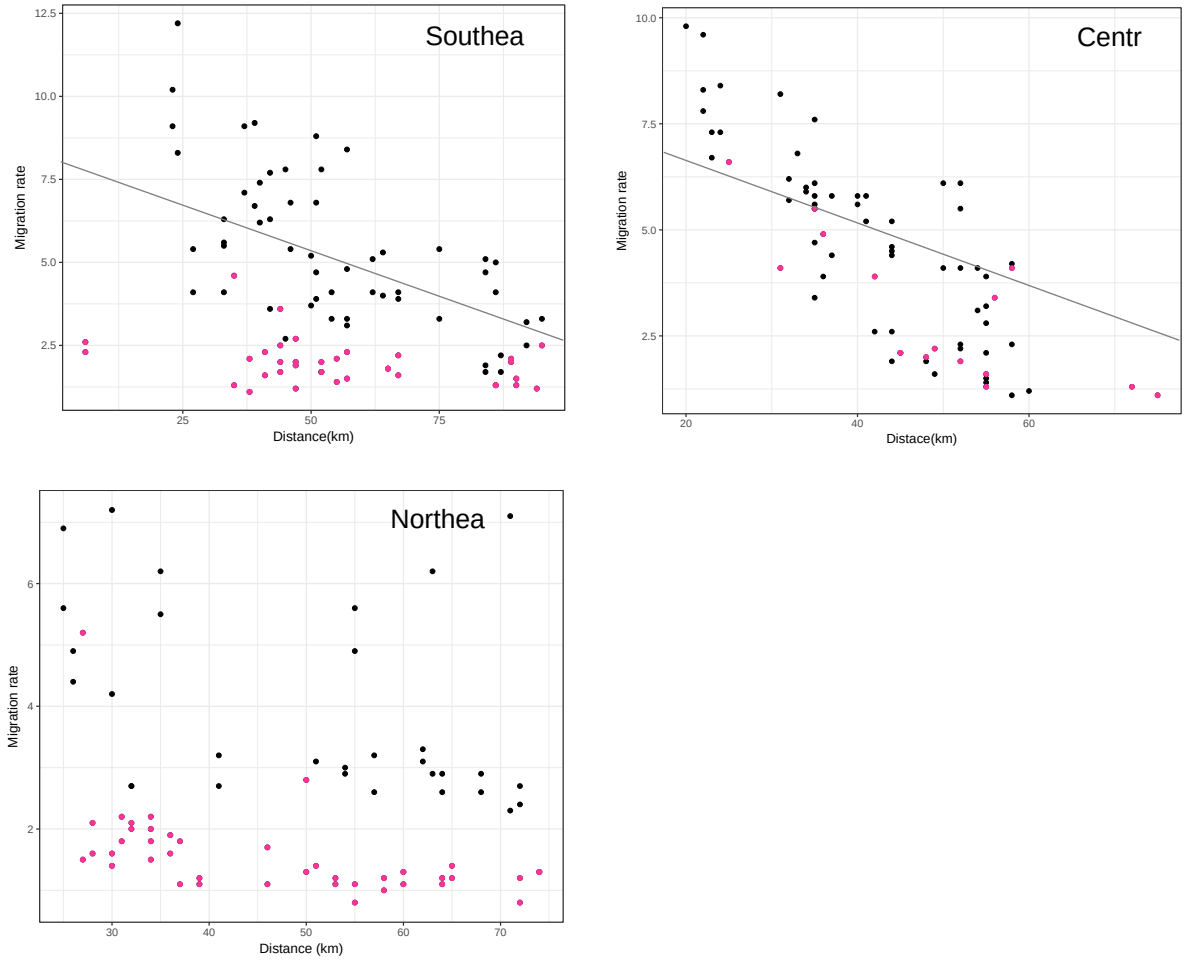


Figure 2.7. Plots of migration rates vs. distance (km) in all three regions. Rates that involved at least one populations with females are pink, hermaphrodite-only populations are black.

Table 2.1. List of all populations used in this survey, including their region of origin, population female frequency, and GPS coordinates.

Population	Region	State	Female frequency	GPS Coordinates
EL	SE	GA	0.03	34.770084, -84.589966
SU	SE	GA	0.34	34.77438, -84.67049
CS	SE	GA	0.02	34.519033, -84.56895
SR1	SE	NC	0	35.42773, -82.9188
SR2	SE	NC	0.32	35.422394, -84.67049
BC	SE	NC	0	35.300683, -83.49055
NR	SE	NC	0.02	35.63237, -83.836633
R113	SE	NC	0	35.3778, -83.683033
GH	SE	NC	0	36.617706, -81.478279
R16	SE	NC	0	36.713154, -81.458328
BCSP	C	VA	0.28	37.532272, -78.274544
BM	C	VA	0.02	38.963985, -78.019646
HO	C	VA	0.03	38.630098, -78.02755
RBR	C	VA	0	39.199177, -78.116249
SSP	C	PA	0.03	40.08662, -78.118034
GP	C	PA	0.03	40.075329, -76.886673
MS	C	PA	0.06	39.893299, -77.484848
PM	C	PA	0	40.16571, -79.265579
LR	C	PA	0	40.148605, -79.22541
LS	NE	PA	0.2	41.514061, -76.25954
TSF	NE	PA	0.18	41.561447, -77.390251
R362	NE	PA	0.01	41.730373, -77.425308
RT	NE	PA	0	42.398388, -76.55735
WD	NE	NY	0.1	42.318363, -76.49527
CG	NE	NY	0	42.548916, -73.914894
SH	NE	NY	0	42.317379, -73.7871
R23	NE	NY	0.08	42.200165, -73.926727
R28A	NE	NY	0.12	41.934635, -74.224831

Table 2.2. Summary statistics for all populations, including means for dimorphic (female and hermaphrodite) and monomorphic (hermaphrodite-only) populations. Abbreviations: He, expected heterozygosity; Ho, observed heterozygosity; Rs, allelic richness; PA, private alleles.

Population	Female frequency	He	Ho	Rs	PA
Total mean		.184 (0.009)	.188 (0.009)	8.17 (0.17)	4.14 (0.23)
Mean, dimorphic		.199 (.013)	.198 (0.010)	7.98 (0.22)	4.88 (0.21)
Mean, monomorphic		.162 (0.011)	0.173 (0.015)	8.46 (0.24)	3 (0.31)
SR1	0	0.165	0.182	9.32	4
BC	0	0.121	0.142	8.76	3
R113	0	0.155	0.167	9.15	3
GH	0	0.106	0.116	7.32	3
R16	0	0.203	0.299	6.95	3
RBR	0	0.171	0.102	8.37	4
PM	0	0.155	0.202	9.23	3
LR	0	0.198	0.189	8.25	2
RT	0	0.111	0.141	9.04	2
CH	0	0.215	0.199	7.65	3
SH	0	0.187	0.169	9.02	3
R362	0.01	0.122	0.137	8.98	5
CS	0.02	0.233	0.219	8.37	4
NR	0.02	0.245	0.2	7.21	5
BM	0.02	0.196	0.183	9.41	4
EL	0.03	0.126	0.143	8.01	6
HO	0.03	0.273	0.256	7.45	4
SSP	0.03	0.186	0.191	6.91	4
GP	0.03	0.213	0.225	7.87	3
MS	0.06	0.149	0.165	8.21	4
R23	0.08	0.178	0.166	8.34	6
WD	0.1	0.241	0.233	7.8	5
R28A	0.12	0.296	0.276	6.56	5
TSF	0.18	0.146	0.166	7.25	6
LS	0.2	0.205	0.219	8.16	5
BCSP	0.28	0.182	0.192	9.45	6
SR2	0.32	0.119	0.135	9.27	6
SU	0.34	0.274	0.255	6.52	5

Table 2.3. Results of a hierarchical analysis of molecular variance (AMOVA), partitioning the variance among individuals, among populations, and among regions. Significance was tested with 999 permutations.

	% Variance	p-value
Among populations (F_{ST})	0.281	0.001
Among populations within region (F_{SR})	0.225	0.001
Among regions (F_{RT})	0.360	0.001

Table 2.4. Migration rates between all populations within the southeast, central, and northeast regions. Migration rates are number of individuals in Pop2 that came from Pop1 in the previous generation.

Southeast			**Central**			**Northeast**		
Pop1	Pop2	M	Pop1	Pop2	M	Pop1	Pop2	M
EL	SU	1.3	BCSP	BM	2.1	LS	TSF	1.6
EL	CS	7.4	BCSP	HO	2	LS	R362	1.8
EL	SR1	7.7	BCSP	RBR	2.2	LS	RT	2
EL	SR2	3.6	BCSP	SSP	1.9	LS	CH	1.8
EL	BC	1.7	BCSP	GP	1.6	LS	WD	0.8
EL	NR	4.1	BCSP	MS	1.3	LS	SH	1
EL	R113	5.4	BCSP	PM	1.3	LS	R23	1.2
EL	GH	1.9	BCSP	LR	1.1	LS	R28A	0.8
EL	R16	1.7	BM	BCSP	2.1	TSF	LS	2.1
SU	EL	4.6	BM	HO	6.6	TSF	R362	2
SU	CS	2.1	BM	RBR	4.1	TSF	RT	1.6
SU	SR1	2.3	BM	SSP	5.5	TSF	CH	1.1
SU	SR2	1.7	BM	GP	4.9	TSF	WD	1.2
SU	BC	2.7	BM	MS	3.9	TSF	SH	1.1
SU	NR	2.1	BM	PM	4.1	TSF	R23	1.1
SU	R113	1.8	BM	LR	3.4	TSF	R28A	1.3
SU	GH	1.3	HO	BCSP	1.9	R362	LS	2.2
SU	R16	1.5	HO	BM	6.8	R362	TSF	2.1
CS	EL	6.2	HO	RBR	6.1	R362	RT	5.5
CS	SU	1.1	HO	SSP	5.9	R362	CH	2.7
CS	SR1	6.8	HO	GP	6.1	R362	WD	3.1
CS	SR2	1.2	HO	MS	5.5	R362	SH	3.3
CS	BC	3.9	HO	PM	3.9	R362	R23	2.9
CS	NR	4.8	HO	LR	4.2	R362	R28A	2.7
CS	R113	5.1	RBR	BCSP	1.6	RT	LS	2.2
CS	GH	4.7	RBR	BM	8.2	RT	TSF	1.9
CS	R16	3.2	RBR	HO	7.6	RT	R362	6.2
SR1	EL	6.3	RBR	SSP	5.6	RT	CH	5.4
SR1	SU	1.6	RBR	GP	5.2	RT	WD	2.8
SR1	CS	5.4	RBR	MS	6.1	RT	SH	3
SR1	SR2	2.3	RBR	PM	4.1	RT	R23	2.6
SR1	BC	3.7	RBR	LR	3.2	RT	R28A	2.9
SR1	NR	3.3	SSP	BCSP	2.2	CH	LS	1.1
SR1	R113	4	SSP	BM	5.8	CH	TSF	1.2
SR1	GH	4.1	SSP	HO	6	CH	R362	3.2
SR1	R16	3.3	SSP	RBR	5.8	CH	RT	2.7
SR2	EL	2.5	SSP	GP	4.6	CH	WD	1.7
SR2	SU	2	SSP	MS	5.8	CH	SH	5.6
SR2	CS	1.9	SSP	PM	5.8	CH	R23	6.2
SR2	SR1	2.6	SSP	LR	4.7	CH	R28A	1.7

SR2	BC	1.7
SR2	NR	1.5
SR2	R113	1.6
SR2	GH	2
SR2	R16	1.2
BC	EL	7.8
BC	SU	2
BC	CS	4.7
BC	SR1	5.2
BC	SR2	2
BC	NR	5.4
BC	R113	5.6
BC	GH	2.7
BC	R16	3.1
NR	EL	3.9
NR	SU	1.4
NR	CS	3.3
NR	SR1	4.1
NR	SR2	2.3
NR	BC	4.1
NR	R113	6.3
NR	GH	9.2
NR	R16	8.8
R113	EL	3.3
R113	SU	1.8
R113	CS	4.1
R113	SR1	5.3
R113	SR2	2.2
R113	BC	4.1
R113	NR	5.5
R113	GH	9.1
R113	R16	12.2
GH	EL	1.7
GH	SU	1.3
GH	CS	5.1
GH	SR1	5
GH	SR2	2.1
GH	BC	7.8
GH	NR	6.7
GH	R113	7.1
GH	R16	10.2
R16	EL	2.2
R16	SU	1.3
R16	CS	2.5
R16	SR1	3.6

GP	BCSP	1.5
GP	BM	3.9
GP	HO	4.1
GP	RBR	4.4
GP	SSP	4.5
GP	MS	5.6
GP	PM	6.2
GP	LR	7.3
MS	BCSP	1.4
MS	BM	2.6
MS	HO	2.3
MS	RBR	4.1
MS	SSP	5.2
MS	GP	5.5
MS	PM	6.7
MS	LR	7.8
PM	BCSP	1.1
PM	BM	2.6
PM	HO	2.8
PM	RBR	3.1
PM	SSP	4.4
PM	GP	5.7
PM	MS	7.3
PM	LR	8.3
LR	BCSP	1.2
LR	BM	1.9
LR	HO	2.3
LR	RBR	2.1
LR	SSP	3.4
LR	GP	8.4
LR	MS	9.6
LR	PM	9.8

WD	LS	1.1
WD	TSF	1.1
WD	R362	1.4
WD	RT	1.3
WD	CH	1.1
WD	SH	1.5
WD	R23	1.6
WD	R28A	1.5
SH	LS	1.2
SH	TSF	1.3
SH	R362	3.1
SH	RT	2.9
SH	CH	4.9
SH	WD	5.2
SH	R23	4.4
SH	R28A	4.2
R23	LS	1.4
R23	TSF	1.2
R23	R362	2.6
R23	RT	3.2
R23	CH	2.9
R23	WD	1.4
R23	SH	4.9
R23	R28A	5.6
R28A	LS	1.2
R28A	TSF	1.3
R28A	R362	2.4
R28A	RT	2.6
R28A	CH	2.3
R28A	WD	1.8
R28A	SH	2.3
R28A	R23	2.5

R16	SR2	2.5
R16	BC	8.4
R16	NR	6.8
R16	R113	8.3
R16	GH	9.1

Table 2.5. Summary statistics with females removed from all populations. Data shown includes hermaphrodite individuals only; female frequency included for reference. Abbreviations: He, expected heterozygosity; Ho, observed heterozygosity; Rs, allelic richness; PA, private alleles.

Population	Female frequency	He	Ho	Rs	PA
Total mean		.179 (0.010)	.185 (0.007)	8.22 (0.20)	3.82 (0.20)
Mean, dimorphic		.189 (.013)	.197 (0.012)	7.97 (0.15)	4.63 (0.17)
Mean, monomorphic		.169 (0.015)	0.173 (0.01)	8.46 (0.24)	3 (0.31)
SR1	0	0.165	0.182	9.32	4
BC	0	0.121	0.142	8.76	3
R113	0	0.155	0.167	9.15	3
GH	0	0.106	0.116	7.32	3
R16	0	0.203	0.299	6.95	3
RBR	0	0.171	0.102	8.37	4
PM	0	0.155	0.202	9.23	3
LR	0	0.198	0.189	8.25	2
RT	0	0.111	0.141	9.04	2
CH	0	0.215	0.199	7.65	3
SH	0	0.187	0.169	9.02	3
R362	0.01	0.121	0.137	8.98	5
CS	0.02	0.222	0.219	8.37	4
NR	0.02	0.239	0.199	7.21	5
BM	0.02	0.189	0.178	9.41	4
EL	0.03	0.12	0.143	8	6
HO	0.03	0.273	0.256	7.45	4
SSP	0.03	0.186	0.191	6.91	4
GP	0.03	0.201	0.225	7.87	3
MS	0.06	0.131	0.155	8.27	4
R23	0.08	0.169	0.172	8.34	5
WD	0.1	0.197	0.233	7.1	5
R28A	0.12	0.231	0.276	6.56	5
TSF	0.18	0.121	0.166	7.25	6
LS	0.2	0.177	0.221	8.03	5
BCSP	0.28	0.199	0.192	9.45	4
SR2	0.32	0.152	0.131	9.51	5
SU	0.34	0.281	0.262	6.78	5

References

Agren, J., and M. F. Willson. 1991. Gender Variation and Sexual Differences in Reproductive Characters and Seed Production in Gynodioecious Geranium maculatum.

Caruso, C. M., and S. J. Yakobowski. 2008. Selection on floral and carbon uptake traits of Lobelia siphilitica is similar in females and hermaphrodites. J. Evol. Biol. 21:1514–1523.

Chang, S.-M. 2006. Female compensation through the quantity and quality of progeny in a gynodioecious plant, Geranium maculatum (Geraniaceae). Am. J. Bot. 93:263–270.

Delph, L. 1990. Sex differential resource allocation patterns in the subdioecious shrub Hebe subalpine. Ecology 71:1342–1351.

Delph, L. F., and M. F. Bailey. 2010. The nearness of you: The effect of population structure on siring success in a gynodioecious species. Mol. Ecol. 19:1520–1522.

Dudle, D. a., P. Mutikainen, and L. F. Delph. 2001. Genetics of sex determination in the gynodioecious species Lobelia siphilitica: Evidence from two populations. Heredity (Edinb). 86:265–276.

Dufay, M., and E. Billard. 2012. How much better are females? the occurrence of female advantage, its proximal causes and its variation within and among gynodioecious species. Ann. Bot. 109:505–519.

Dufay, M., J. Cuguen, J.-F. Arnaud, and P. Touzet. 2009. Sex ratio variation among gynodioecious populations of sea beet: can it be explained by negative frequency-dependent selection? Evolution 63:1483–97.
Earl, D. 2011. Structure Harvester v.0.6.1.

Emery, S. N., and D. E. McCauley. 2002. Consequences of inbreeding for offspring fitness and gender in Silene vulgaris, a gynodioecious plant. J. Evol. Biol. 15:1057–1066.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. 14:2611–2620.

Fénart, S., P. Touzet, J.-F. Arnaud, and J. Cuguen. 2006. Emergence of gynodioecy in wild beet (Beta vulgaris ssp. maritima L.): a genealogical approach using chloroplastic nucleotide sequences. Proc. Biol. Sci. 273:1391–8.

Goudet, J. 1995. FSTAT (Version 1.2): A computer program to calculate F-statistics. J. Hered. 86:485–486.

Hanson, M. R. 1991. Plant mitochondrial mutations and male sterility. Ann. Rev. Genet. 24:461–486.

Hodgins, K. a, and S. C. H. Barrett. 2008. Natural selection on floral traits through male and female function in wild populations of the heterostylous daffodil Narcissus triandrus. Evolution 62:1751–63.

Hodgins, K. a, and S. C. H. Barrett. 2007. Population structure and genetic diversity in tristylous Narcissus triandrus: insights from microsatellite and chloroplast DNA variation. Mol. Ecol. 16:2317–32.

JJ, D., and J. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus (Madison). 12:13–15.

Lloyd, D. G. 1974. Theoretical sex ratios of dioecious and gynodioecious angiosperms. Heredity (Edinb). 32:11–34.

McCauley, D. E., and M. F. Bailey. 2009. Recent advances in the study of gynodioecy: the interface of theory and empiricism. Ann. Bot. 104:611–20.

Mccauley, D. E., and M. T. Brock. 1998. frequency-dependent fitness in Silene vulgaris, a gynodioecious plant. Evolution (N. Y). 52:30–36.

McCauley, D. E., and M. S. Olson. 2008. Do recent findings in plant mitochondrial molecular and population genetics have implications for the study of gynodioecy and cytonuclear conflict? Evolution 62:1013–25.

Mccauley, D. E., and D. R. Taylor. 1997. Local population structure and sex ratio: evolution in gynodioecious plants. Am. Nat. 150.

Olson, M. S., D. E. McCauley, and D. Taylor. 2005. Genetics and adaptation in structured populations: Sex ratio evolution in Silene vulgaris. Genetica 123:49–62.

Peakall, R., and P. E. Smouse. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research--an update. Bioinformatics 28:2537–9.

Pérez-Alquicira, J., F. E. Molina-Freaner, D. Piñero, S. G. Weller, E. Martínez-Meyer, J. Rozas, and C. a Domínguez. 2010. The role of historical factors and natural selection in the evolution of breeding systems of Oxalis alpina in the Sonoran desert “Sky Islands”. J. Evol. Biol. 23:2163–75.

Pritchard, J., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Sloan, D. B., K. Muller, D. E. McCauley, D. R. Taylor, and H. Storchova. 2012. Intraspecific variation in mitochondrial genome sequence , structure , and gene content in Silene vulgaris , an angiosperm with pervasive cytoplasmic male sterility. 1228–1239.

Van Etten, M. L., and S. M. Chang. 2014. Frequency-dependent pollinator discrimination acts against female plants in the gynodioecious Geranium maculatum. Ann. Bot. 114:1769–1778.

Zhou, W., S. C. H. Barrett, H. Wang, and D. Z. Li. 2012. Loss of floral polymorphism in heterostylous luculia pinceana (rubiaceae): A molecular phylogeographic perspective. Mol. Ecol. 21:4631–4645.

CHAPTER THREE

PATTERNS OF ALLOCATION TO REPRODUCTIVE STRUCTURES IN FEMALES AND HERMAPHRODITES IN NATURAL POPULATIONS OF THE GYNODIOECIOUS *GERANIUM MACULATUM*.[1]

[1]Christopher, Dorothy A. and S.-M. Chang. To be submitted to *Annals of Botany*.

Abstract

Gynodioecy, in which females and hermaphrodites co-occur in a species, is thought to be an intermediate stage in the evolution of separate male and female sexes (dioecy) from a hermaphroditic ancestor. Here the increased availability of ovules from females puts selective pressure on hermaphrodites to increase allocation to male function at the expense of ovule production, which facilitates the invasion of a female sterility mutation. We tested the first part of this theoretical prediction in 10 natural populations of *Geranium maculatum* that vary from 0-34% female, expecting the hermaphrodites should show increased allocation to male traits when female frequency is high. We measured a suite of traits related to male and female function. We did not find evidence that hermaphrodites were "more male" at high female frequency: pollen production was not significantly different. This suggests dioecy is unlikely to evolve in this system. Additionally, we did not find evidence of female compensation in seed production, which is required for females to be maintained. However, the rhizomes of females are larger, suggesting that they may have higher lifetime fitness than hermaphrodites.

Introduction

Plants exhibit a striking lability in sexual function, with male and female function partitioned in a variety of ways between individuals. Most common is dioecy, with separate male and female individuals, and gynodioecy, in which female and hermaphrodites co-occur in a species. These sexual systems occur across the angiosperm phylogeny; they are found in more than 40% of angiosperm families, suggesting that these sexual systems must provide a selective advantage under conditions common among many plant species (Renner 2014). While sexual system evolution has been studied since Darwin (1877), we still do not entirely understand the population dynamics and conditions that facilitate the establishment of separate sexes. Most studies in gynodioecious systems focus on the requirements necessary to maintain females, but we lack an understanding of how hermaphrodites acquire fitness and how the presence of females influences hermaphrodite fitness.

The evolutionary pathway to dioecy that has received the most theoretical support posits that dioecy evolved from hermaphroditism through a gynodioecious intermediate stage (Lloyd 1974; Charlesworth and Charlesworth 1978). In the first stage of the evolution of dioecy, a male sterility mutation that renders a plant female invades a hermaphroditic population. Hermaphrodites can gain fitness through pollen and seed production, but as females can only acquire fitness through seeds, they are at a fitness disadvantage. Thus females will establish in the population only if they can compensate for their lost siring ability. They must increase female function, which can be accomplished through having higher seed fitness, either through higher quantity or quality of seeds, than hermaphrodites (Charlesworth and Charlesworth 1978; Charlesworth and Ganders 1979).

There are three mechanisms females can use to compensate for their loss of male function (Lloyd 1974). First, female compensation can occur through the mating system. Females necessarily outcross, while hermaphrodites of gynodioecious species are self-compatible and thus can self (Charlesworth 1979). The higher selfing rates of hermaphrodites potentially results high inbreeding depression, which can reduce the fitness of hermaphrodite progeny. Indeed, a meta-analysis by Collin and Shykoff (2003) reveals that, of the twenty species studied, all hermaphrodites self, and 45% have selfing rates greater than 0.50. In this scenario, females can compensate by producing better quality progeny that do not suffer from inbreeding depression. Second, females can compensate through seed production; they can increase the number of seeds produced to equal the fitness gained by hermaphrodites through siring. Most females show increased seed production relative to hermaphrodites (see reviews in Dufay and Billard (2012) and Shykoff et al. (2003)). Finally, females can allocate additional resources to seeds to yield better quality progeny. For example, females of the species *Hebe subalpina* allocate more than four times as much biomass to fruits than do hermaphrodites (Delph 1990). In *Leucopogon melaleucoides*, female fruit set increased proportionally with flower number, whereas hermaphrodite fruit set was negatively affected by flower number, suggesting that females allocate more resources to fruit production (Vaughton and Ramsey 2011). These three mechanisms provide the means by which females can successfully compete with hermaphrodites and persist in populations. The population can either remain in a gynodioecious state, or dioecy can evolve.

For dioecy to evolve, there are a set a theoretical predictions that must be met. Hermaphrodites are expected to become “more male” in their functional gender as female frequency increases: hermaphrodites will gain more of their fitness through male function. This

is because the presence of females increases the number and availability of ovules in the population. This creates selective pressure that favors hermaphrodites with higher pollen production, and if this is coupled with a decrease in ovule and/or seed production, we would expect that hermaphrodites will become functionally and structurally more male (Spigler and Ashman 2012). This could create conditions in which a female sterility mutation, rendering a hermaphrodite individual male, could, in principle, enter the population and be maintained (Dorken and Mitchard 2008). The population then becomes trioecious, with females, males, and hermaphrodites coexisting. Eventually the hermaphrodites will be outcompeted because males and females would have higher pollen and seed fitness, respectively, than hermaphrodites, resulting in a dioecious population (Ross 1978, 1982).

Yet there are few studies that have examined the gynodioecy-dioecy transition, particularly the dynamics of this shift in natural populations. Most of research that has been performed has utilized experimental arrays (McCauley and Brock 1998; Ehlers and Thompson 2004). However, we lack an understanding of how individuals allocate to male and female traits in natural populations. One feature of gynodioecious species makes a study in natural populations possible: inter-population sex ratio is highly variable (e.g. *Lobelia siphilitica* (Caruso and Case 2007), *Daphne laureola* (Alonso and Herrera 2011), *Thymus vulgaris* (Landergott et al. 2009)). If a positive correlation is found between male traits and female frequency, this might indicate that selection has acted to increase male function in hermaphrodites. We can then use this information to make predictions regarding the evolutionary trajectory of gynodioecy in a species.

To that end, in this study we used the sex ratio variation found across ten populations of *Geranium maculatum* to examine how floral traits in hermaphrodites and females vary

depending on the female frequency. This study examines factors proposed to be important across different stages of the transition from hermaphroditism to dioecy pathway, including the female compensation necessary to maintain females and the increased allocation to male traits in hermaphrodites that precedes the evolution of dioecy. Specifically, we ask the following questions: 1) Is there variation in the floral traits of hermaphrodites and females between populations? 2) Is there evidence of female compensation through seed production? and 3) Do hermaphrodites increase allocation to traits important for male fitness as female frequency increases?

Methods

Study system

Geranium maculatum is an understory perennial herb found across eastern North America, from southern Canada south to Georgia and east to the Mississippi River. Plants leaf out and flower in early spring, producing one inflorescence per individual (personal observation). Flowers are generalist pollinated, but the majority of pollinators are small bees (Van Etten and Chang). Seeds mature in early summer; seed dispersal occurs by dehiscence of the elastic schizocarp. The aboveground structure dies back in the fall and the rhizome overwinters.

G. maculatum is gynodioecious and sex ratio is highly variable among populations. Female frequency varies from 0-40%; among dimorphic (both sexes present) populations, the average frequency is 11%. Females can be visually identified by their smaller flowers and aborted anthers. In hermaphrodites, there are ten anthers total that are arranged into two whorls; dehiscence of each whorl is temporally separated, but there is a period of overlap between the two whorls.

Population sampling

This study was performed in 2014, 2015, and 2016; in all three years it commenced in mid-April when at least two flowers on all sampled inflorescences were blooming. In 2014, we located ten populations in north Georgia and western North Carolina that varied in the female frequency: three populations were hermaphrodite-only; four populations had low female frequency, ~5%; and three populations had high female frequency, 30-35% (Fig. 3.1 and Table 3.1). We obtained the sex ratio for each population by counting the number of female and hermaphrodite individuals flowering in each population. Females can be visually identified by their smaller flower size and the presence of aborted anthers that lack pollen. The yearly census showed that the populations' female frequencies remained relatively consistent over the three years of the study.

Trait measurements

In each population, we measured floral and vegetative traits for every inflorescence stalk. *G. maculatum* plants have an underground rhizome with an inflorescence emerging from one end. Measurements were performed on all inflorescences for both hermaphrodites and females in the population. We measured the following floral traits: the number of inflorescences; the total number of flowers per inflorescence, which was calculated as the sum of the buds and flowers present on the day of data collection; and the size of two flowers per inflorescence stalk by taking two perpendicular measurements per flower. We collected pollen from newly dehisced anthers. We collected all ten anthers from flowers in which both whorls were dehisced that showed no sign of pollen removal by pollinators. Anthers from each flower were placed into individual microcentrifuge-tubes with 750ul of 70% ethanol added as a preservative.

In the lab, we used a coulter counter (Beckman Multisizer II) to count and size pollen grains. Using the machine-counted pollen number in the subsample processed, we back-calculated to estimate pollen number for all ten anthers per flower. This method has been shown to provide an accurate estimate of the total pollen number (S.-M. Chang, unpublished data).

Following the completion of flowering for the entire inflorescence, immature fruits were bagged and the tops of the fruits were snipped to prevent the schizocarp from dehiscing before we were able to collect them. This method was tested previously and shown not to affect seed set (Van Etten and Chang 2015). In the field, we counted the number of fruits produced . Seeds were collected when mature and counted in the lab, approximately three weeks after the end of the flowering.

In 2015 and 2016, we measured the length of the rhizome of every flowering individual. *Geranium maculatum* rhizomes grow shallowly below the soil surface, so measuring the rhizome length simply involved brushing the soil off the rhizome to allow measuring and then replacing the soil afterward.

Data analysis

We used a generalized linear mixed model (GLMM) to test whether the floral traits of hermaphrodites and females varied with the population sex ratio. Population was coded as a random effect; female frequency of the population was a continuous variable. Data that were proportions, including the proportion of flowers setting fruit and the proportion of fruits setting seed, were arcsin square root transformed to satisfy the normality assumption of the analysis. Count data such as total flower number and pollen production were analyzed with a negative binomial distribution.

For pollen production, we took the average of the two flowers analyzed for pollen production to obtain a single value each for pollen production and average pollen size per plant. We then took the average of all individuals in a population, and then performed the GLMM on these averaged values. Additionally, we performed a GLMM on the variance in pollen size and pollen number across populations. For all analyses in which the entire model was significant, we then performed a multiple comparisons test to evaluate which population(s) were significantly different.

We quantified female compensation in seed number following the calculation in Chang (2006), $FC_s = (W_{Fs}/W_{Hs})-1$, where W_{Fs} and W_{Hs} were the averages of seed number for females and hermaphrodites, respectively.

We analyzed the data for each year separately (not shown), but as the patterns were qualitatively similar across years, data from all years were pooled into one combined analysis that is presented in this report. We performed all analyses using the *lme4* package in R v.3.2.3 (Pinheiro et al. 2015).

Results

There were significant differences in total flower production between populations, however this was not correlated with the sex ratio (Fig. 3.2; $F_{9,1341}=2.65$, $p=0.006$). The flower production between the two sexes within a population did not differ (Fig. 3.2; $F_{14,1236}=3.10$, $p=0.25$). The number of fruits per flower was not significantly different between sexes or between populations (Fig. 3.3; $F_{4,1306}=1.08$, $p=0.420$).

We found a difference among populations in the number of seeds per fruit. Hermaphrodites in hermaphrodite-only populations showed more seeds per fruit (Fig. 3.4;

$F_{16,1375}$=1.87, p=0.027). Two populations showed a within-population difference in seed set between the two sexes. In populations SU (30% female) and TE (34% female), females produced significantly more seeds than hermaphrodites (Fig 3.4; SU, $F_{1,73}$=1.09, p=0.016; TE, $F_{1,61}$=3.43, p=0.011). Seed number per plant showed a similar pattern (Fig. 3.5; $F_{4,1367}$=1.04, p<0.01). In two hermaphrodite-only populations (CH and TP), individuals produced more seeds than individuals in populations with females (Fig 3.5; CH, $F_{16,1373}$=3.42, p=0.03; TP, $F_{16,1374}$=2.91, p=0.028). In populations SU and TE, females produced more seeds than hermaphrodites (Fig 3.5; SU, $F_{1,75}$=2.78, p<0.01; TE, $F_{1,63}$=3.54, p<0.001).

An average of 6745±245 pollen grains were produced per flower, with an average size of 78±9.2 microns. Overall, pollen production per flower did not vary with population female frequency (Fig 3.6a; R^2=-0.11, p=0.77). However, mean pollen size decreased as the female frequency increased in the populations (Fig. 3.6b; R^2=-0.34, p=0.037). Interestingly, the variance in pollen grain number and size showed the opposite pattern; the variance of these two traits increased with the population female frequency (number, Fig. 3.7a, R^2=0.49, p=0.008; size, Fig. 3.7b, R^2=0.56, p=0.007).

Females had significantly longer rhizomes than their hermaphrodite counterparts in four out of the seven populations in which both sexes were present (Fig. 3.8; BH, $F_{1,202}$=3.12, p<0.01; SU, $F_{1,124}$=0.98, p=0.02; SR2, $F_{1,186}$=1.73, p,0.01; TE, $F_{1,169}$=2.02, p=0.03); this includes all of the high female frequency populations (SU, SR2, and TE). Female compensation for seed number, $FC_s = (W_{Fs}/W_{Hs})-1$, did not show a correlation between the compensation value and the population female frequency (Fig. 3.9; R^2=0.39, p=0.08).

Discussion

The objective of this study was to determine how females and hermaphrodites of *G. maculatum* acquire fitness and allocate to reproductive structures. Specifically, we investigated the variation in floral traits among ten natural populations that range from 0-35% female to identify female compensation patterns and to determine how hermaphrodites acquire fitness when females are present. Overall, we did not find overwhelming evidence to suggest either sex meaningfully alters allocation patterns when female frequency is high. This suggests that the populations studied here will remain gynodioecious or will revert to hermaphroditism.

Female compensation

Assuming nuclear control of male sterility, theoretical models suggest that females must produce twice the number of seeds as hermaphrodites to be maintained in the population (Lewis 1941; Charlesworth and Charlesworth 1978). In the seven populations of this study in which females occurred, female compensation in seed number was low, with an average value of 0.08. While the highest FC values were found in two of the high female frequency populations, their values of 0.21 and 0.20 are much lower than what is theoretically necessary to maintain females. Similarly, Chang (2006) calculated seed number FC in two populations in Georgia and found an average of 0.30. This is higher than the FC found in this study, but is nevertheless lower than what we would predict for the stable coexistence of both sexes.

There are two possible explanations for this lack of sufficient FC in this gynodioecious species. The first is that females will not persist over long term time periods in these populations. Male sterility is a large mutational target—there are many ways to arrest normal anther development—perhaps male sterility arises in populations relatively frequently, but the

two sexes are not in equilibrium and females are not maintained long term. For the second explanation, we turn to the life history of *G. maculatum*.

G. maculatum is a long-lived perennial species, and while the conventional approach is to use seed number when evaluating female compensation, this metric alone may not be sufficient for fitness of a long-lived perennial species. This is because growth and stasis could be equally as important as fecundity in any given year for the long-term fitness of a perennial plant (Silvertown and Charlesworth 2009). For example, demographic modeling of *G. maculatum* (Van Etten and Chang, unpublished data) showed that females will be maintained at high frequencies, and this depends on adult survival. This study also found that females flowered more often, resulting in higher lifetime seed production. Interestingly, we found that female rhizome length was greater than the hermaphrodites' rhizome length in high female frequency populations; perhaps enhanced investment in rhizome growth is the mechanism by which females can increase survivorship. This suggests that females in these populations may have an advantage over hermaphrodites in terms of longevity or resources available to flower in stressful conditions, and thus are able to be maintained despite the low level of female compensation.

The populations with the highest female seed production were also the ones with largest female rhizomes, and these were also the populations in which female frequency was highest (~30%). This might suggest that it is these two factors together that allow females to persist at higher frequencies—compensation in seed production alone is not sufficient.

Hermaphrodite allocation to reproductive structures

We found a significant negative relationship between population female frequency and pollen grain size, and there was a significant positive relationship between female frequency and

both pollen number variance and pollen size variance. This was the opposite of what we predicted. We predicted that hermaphrodites would become "more male" as female frequency increased, and thus we should see a positive relationship between the female frequency and the pollen size, and a negative relationship between variance and frequency. The latter is because we expect selection to have reduced variation to maximize fertilization during pollen competition. Instead, the positive relationship suggests that individuals are responding to low nutrient availability.

Delph (1991) described the sex differential plasticity hypothesis (SDP), in which females are predicted to establish in harsh environmental conditions. This is because hermaphrodites will lower seed production when stressed, because seeds are costly to produce, while female seed production will remain the same. Hermaphrodites can make up the lost fitness through siring success, but females cannot, thus they do not show a plastic response to stress. The pollen number and size data found in this study potentially offer support to the SDP. The high female frequency populations are perhaps in harsh abiotic conditions, which limits the resources hermaphrodites have available to allocate to pollen production.

In fact, when plant size, a trait that often varies with resource availability, is used as a covariate in the pollen analyses, the negative correlation disappears (size, $R^2 = -0.10$, $p = 0.122$; number variance, $R^2 = -0.19$, $p = 0.07$; size variance, $R^2 = 0.20$, $p = 0.08$, data not shown). The implications from this are twofold: First, it suggests that hermaphrodites are more resource-limited in populations with higher female frequencies and pollen fitness and plant size decline accordingly. The populations in this study are found in forest understory, a typical habitat for *G. maculatum*, and light may be the limiting factor, although more work is needed to understand the abiotic conditions in these populations. Second, this suggests that stressful

environmental conditions may facilitate female establishment, allowing female frequency to rise in these populations.

Implications for sexual system evolution in Geranium maculatum

If we agree that gynodioecy is the intermediate step between hermaphroditism and dioecy, there are three possible evolutionary outcomes for a gynodioecious species: it can remain stable in gynodioecy, revert to hermaphroditism, or evolve dioecy. For dioecy to evolve, a necessary precursor is that hermaphrodites should become functionally more male to facilitate the establishment of a female sterility mutation. In this study, we did not find hermaphrodites increase their male function as the proportion of females increases in the population. In fact, we found that pollen fitness seems to decline in high female frequency populations.

Additionally, females in these populations showed a small amount of female compensation in seed production, but this was substantially less what is theoretically required for females to persist. Additionally, female frequency in *G. maculatom* populations is typically low: the average, across thirty populations located across the eastern US is 10% (Christopher and Chang, unpublished data). This potentially indicates that most populations are not able to sustain high female frequencies because the level of compensation is simply not high enough. Therefore, strong enough selective pressure is not present in gynodioecious *G. maculatum* populations to push hermaphrodites to change allocation patterns to become male-biased, as the theory suggests.

Results of this study indicated that there is no evidence that hermaphrodites in gynodioecious populations of *G. maculatum* are under selection to become more male-biased when female frequency is high. We are, therefore, led to believe that the populations we have

studied will either remain gynodioecious with female and hermaphrodites coexisting or reverting back to entirely hermaphroditic.

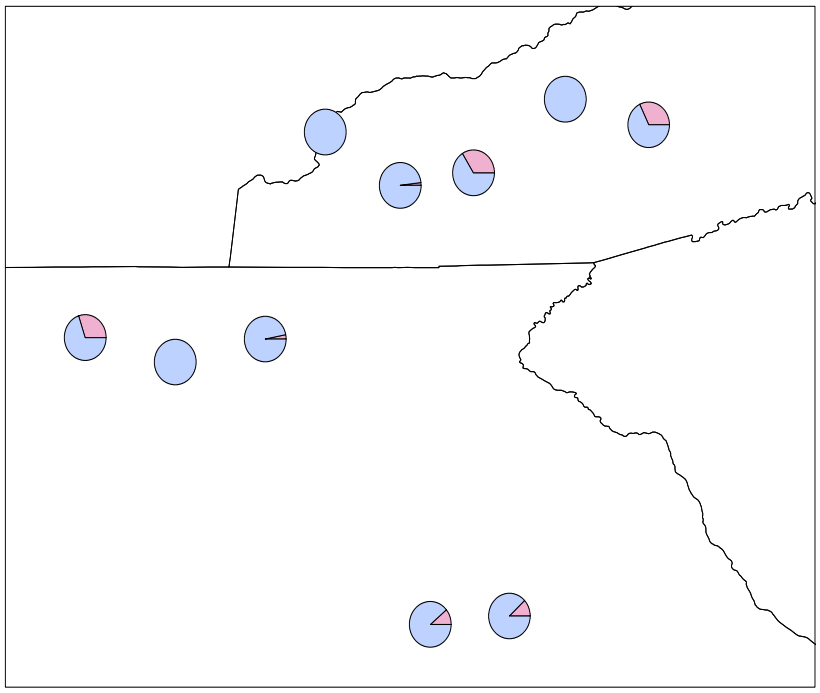


Fig. 3.1. Location of sampled populations. Blue and pink indicate the proportion hermaphrodite and female, respectively.

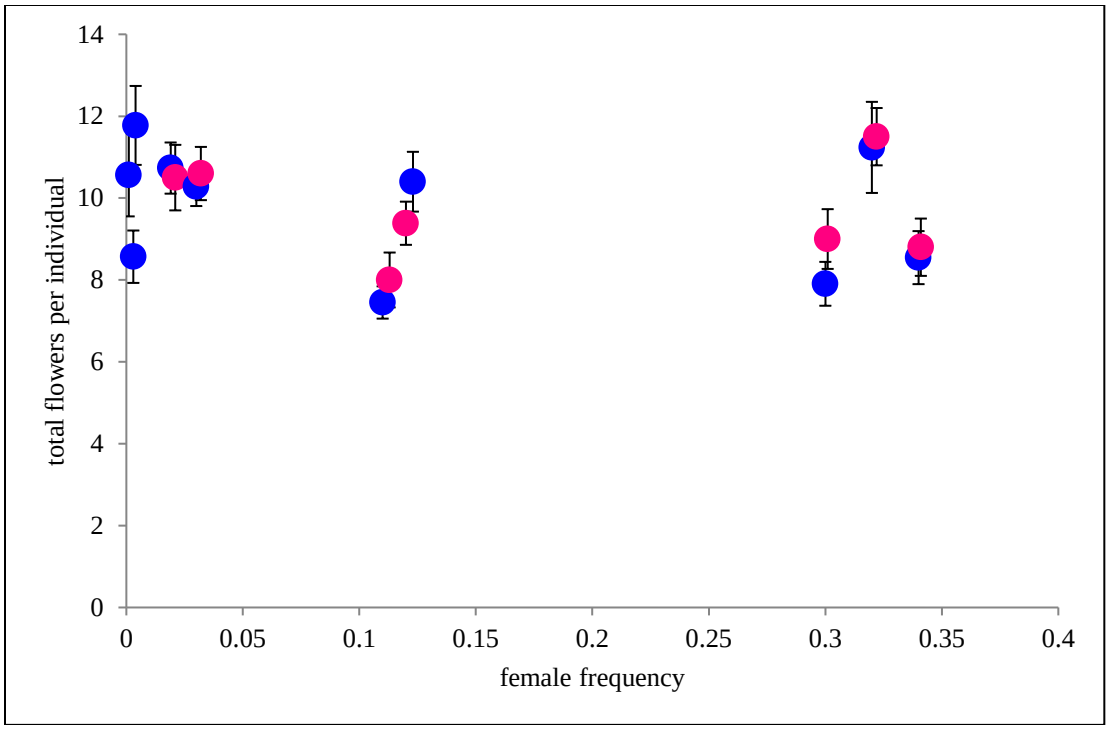


Fig. 3.2. Relationship between total flower production and population female frequency. Blue, hermaphrodite individuals; pink, female.

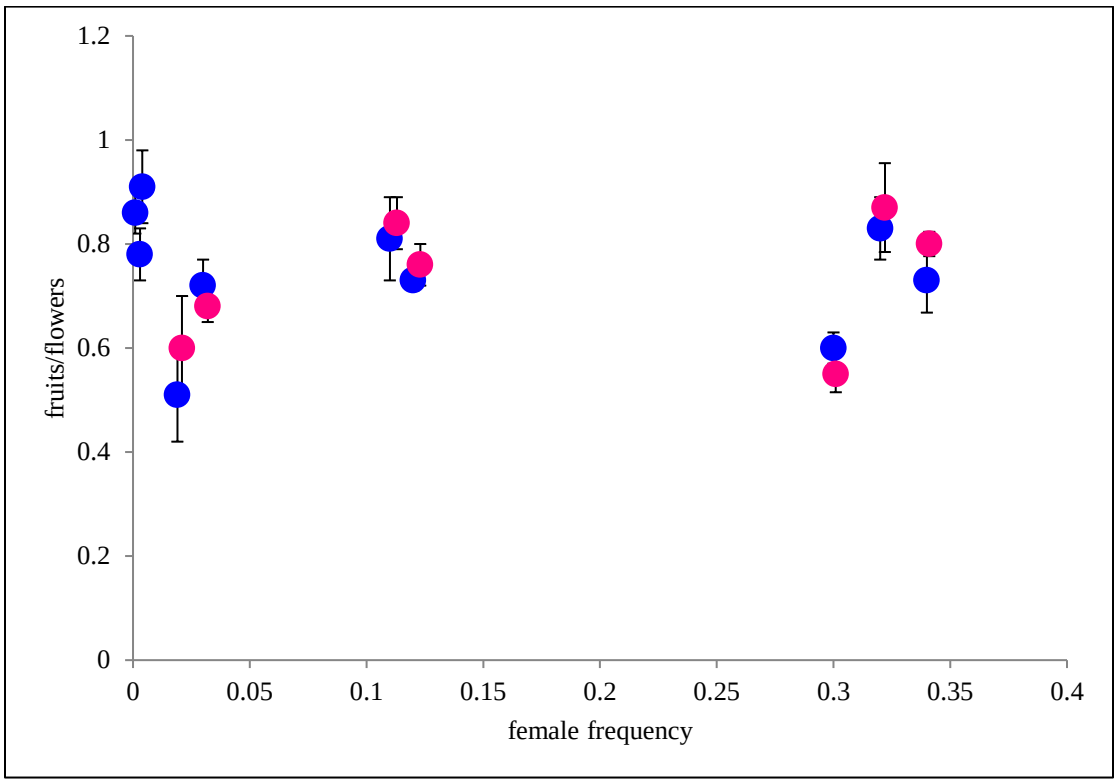


Fig. 3.3. Relationship between the fruit set and the population female frequency. Blue, hermaphrodite individuals; pink, female.

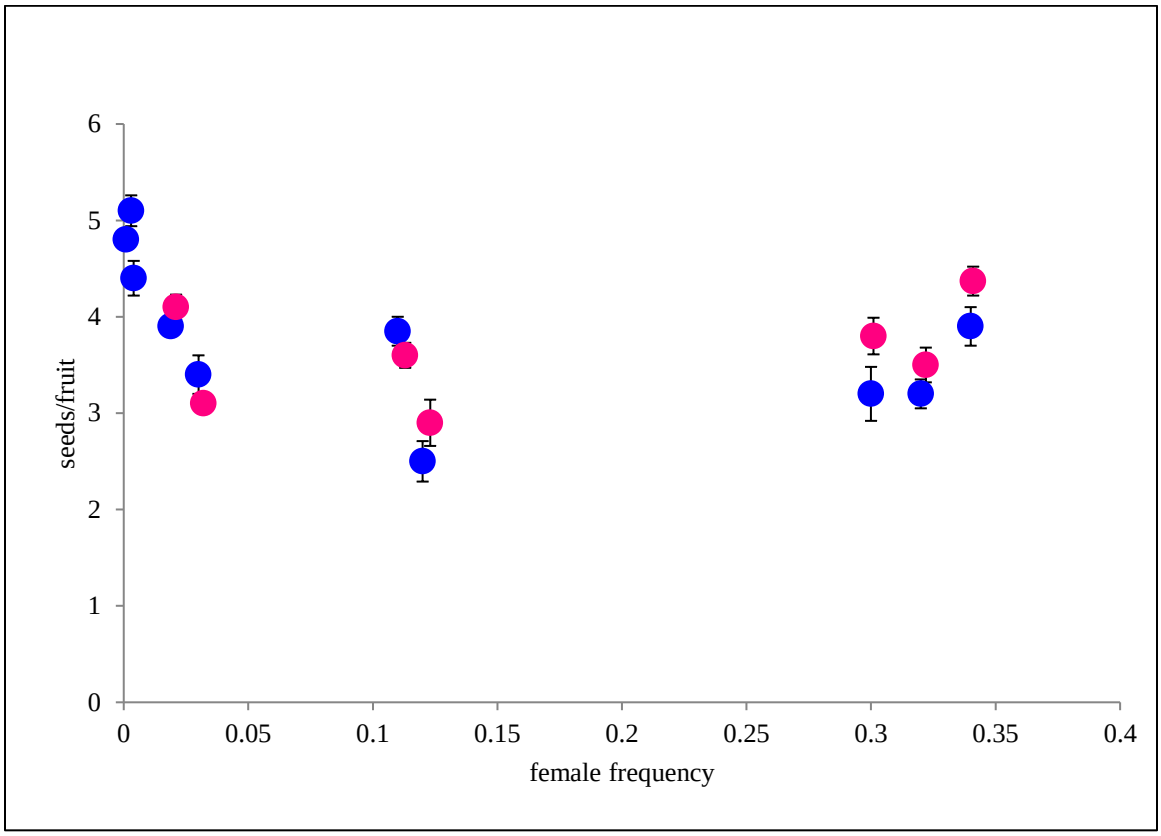


Fig. 3.4. Relationship between seed set and the population female frequency. Blue, hermaphrodite individuals; pink, female. Statistically significant differences at the $p<0.05$ level noted with different letters.

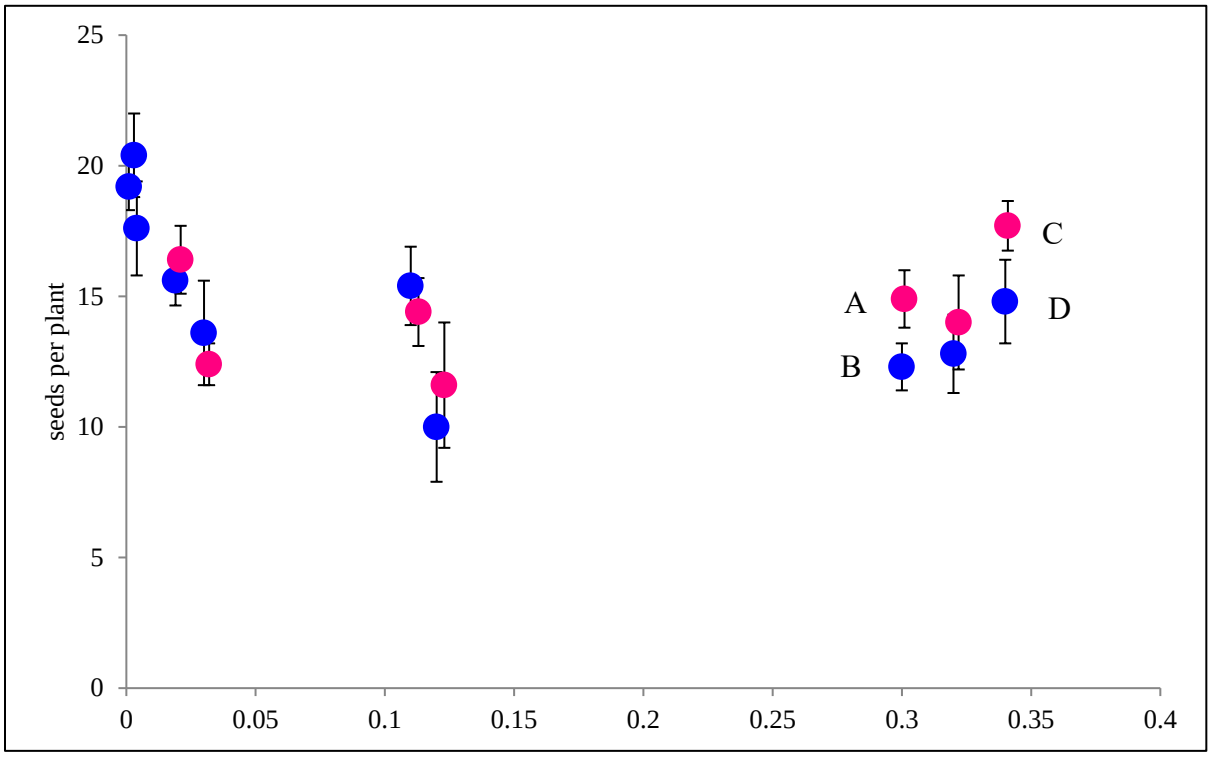
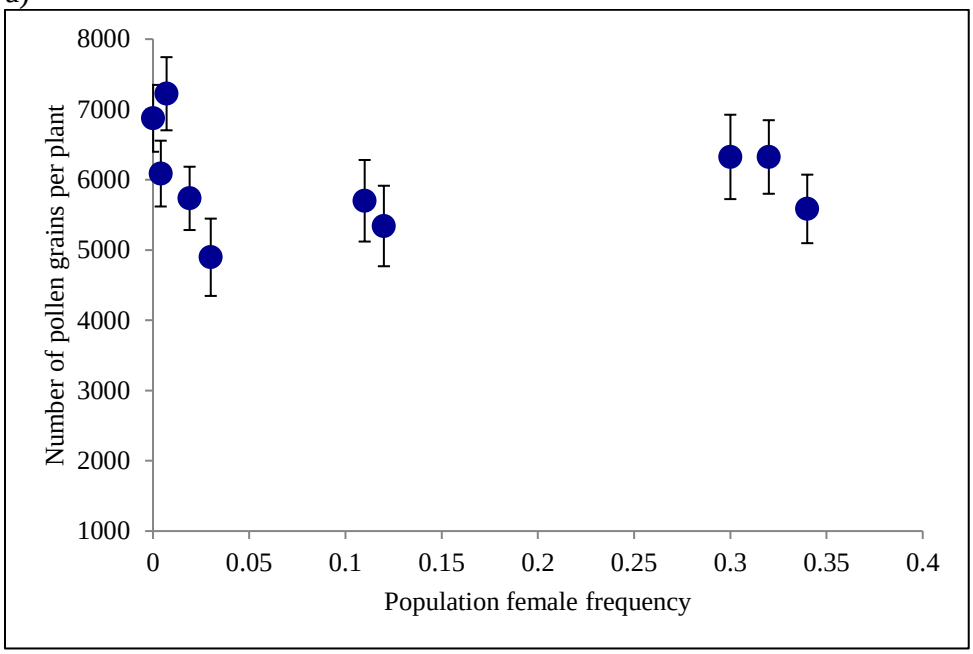


Fig. 3.5. Relationship between total seeds produced per plant and the population female frequency. Blue, hermaphrodite individuals; pink, females. Statistically significant differences at the $p<0.05$ level noted with different letters.

a)



b)

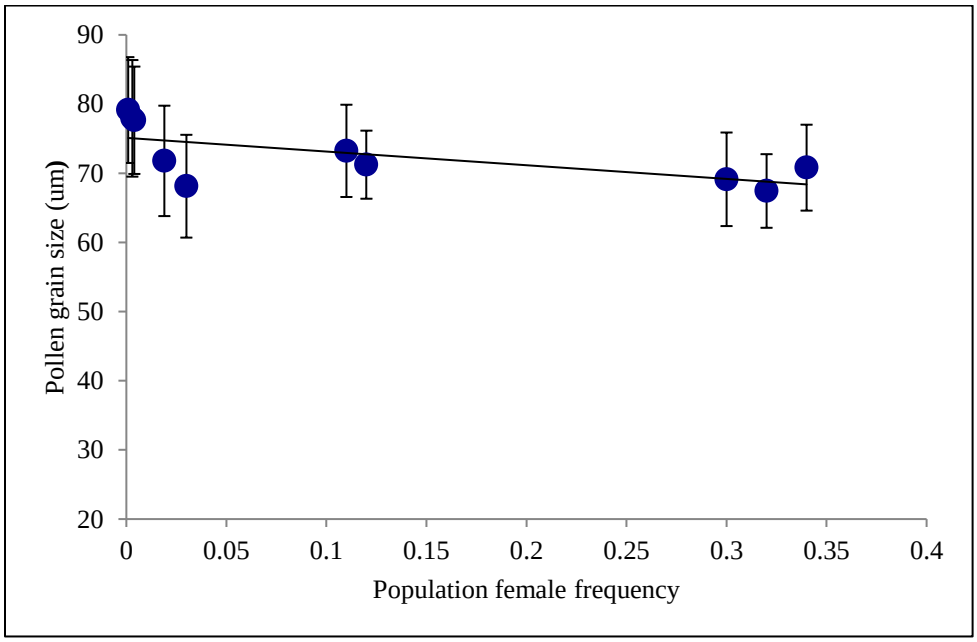
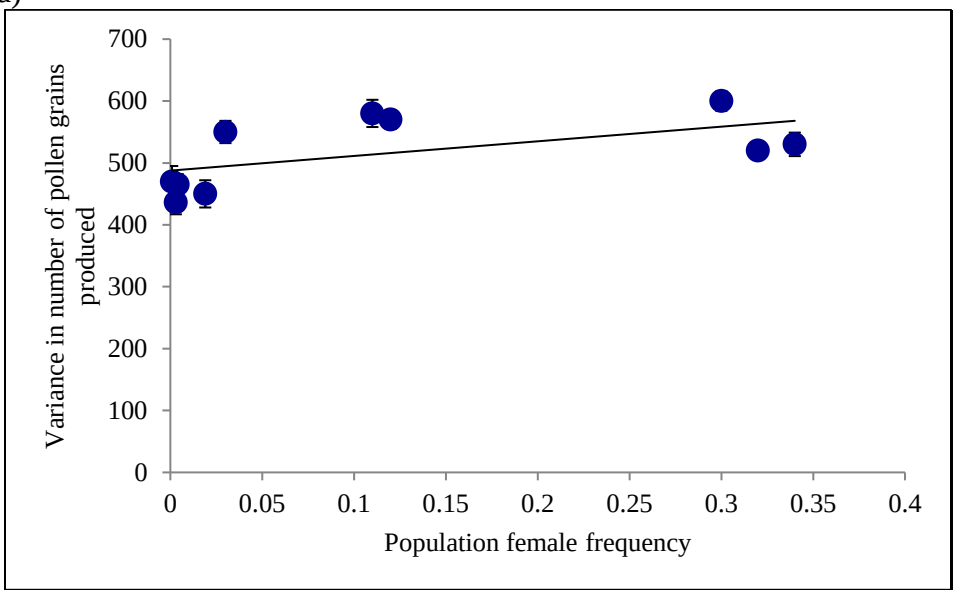


Fig. 3.6. Relationship between a) number of pollen grains produced per hermaphrodite and b) the pollen grain size and the population female frequency.

a)



b)

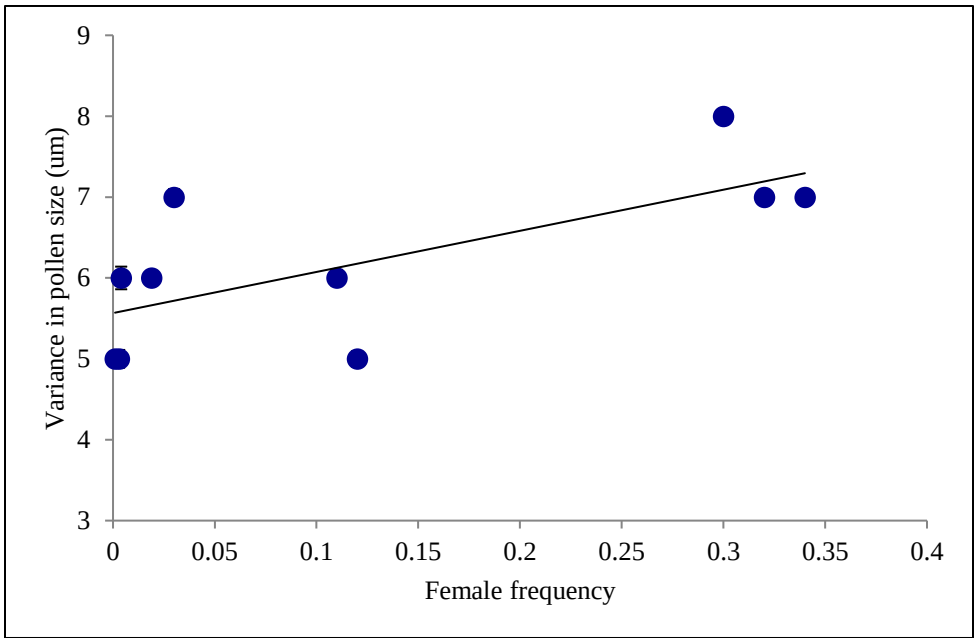


Fig. 3.7. Correlation between the a) variance in pollen grain number and b) variance in pollen grain size and the population female frequency.

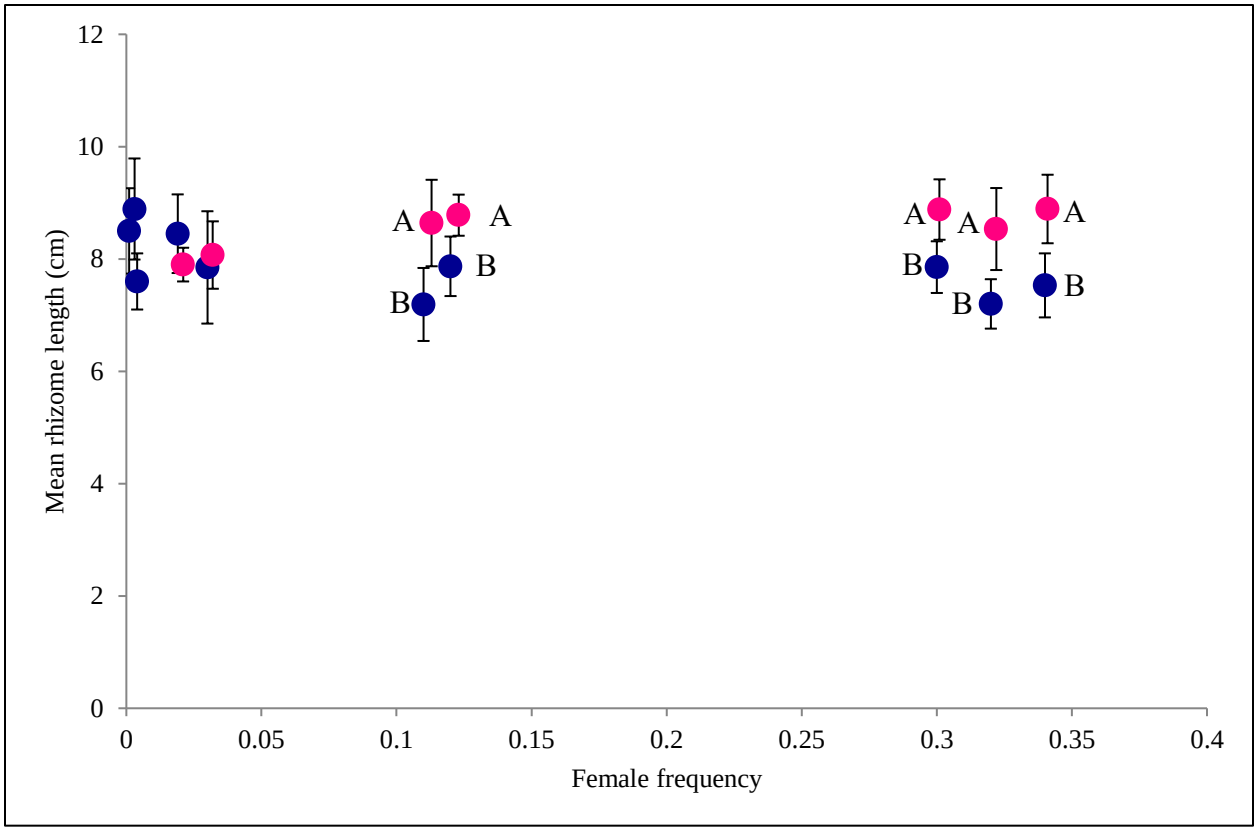


Fig. 3.8. Relationship between rhizome length in hermaphrodites (blue) and female (pink) and the population female frequency. Different letters indicate means are significantly different at the $p<0.05$ level.

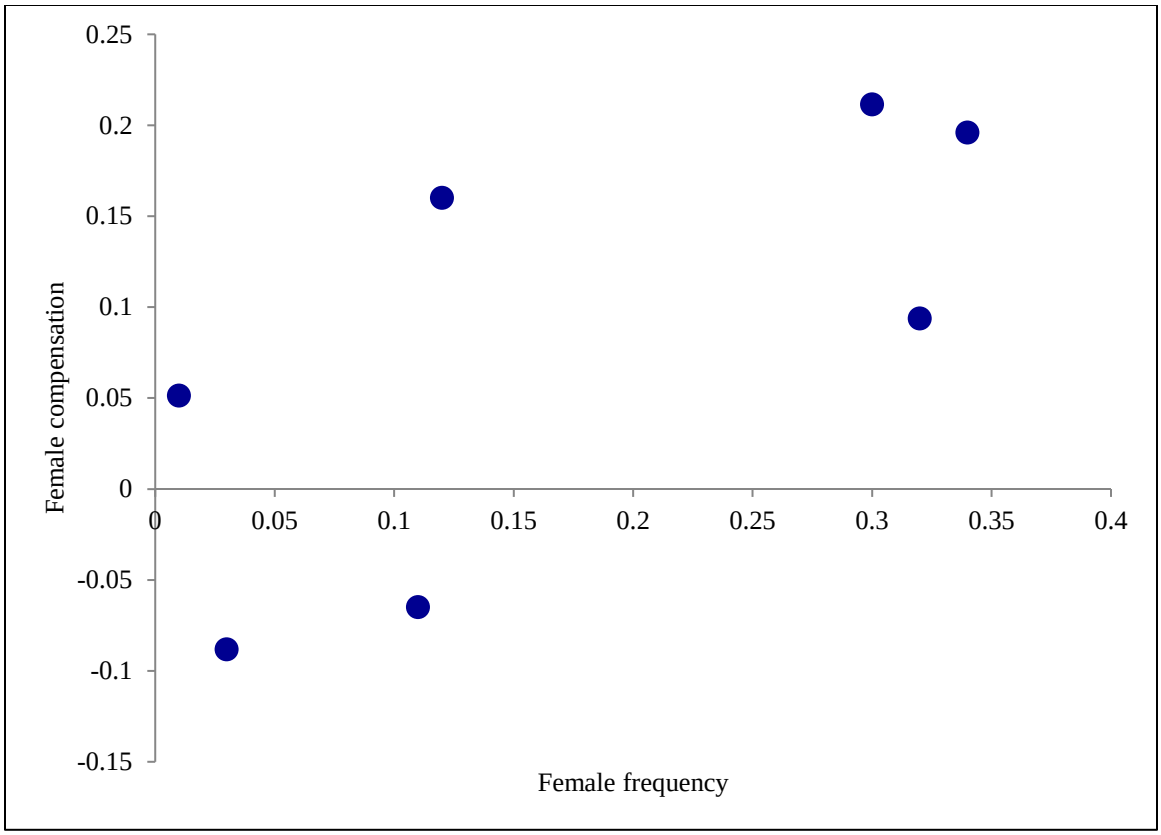


Fig. 3.9. Female compensation in seed number plotted against the population female frequency.

References

Alonso, C., and C. M. Herrera. 2011. Back-and-forth hermaphroditism: Phylogenetic context of reproductive system evolution in subdioecious Daphne laureola. Evolution (N. Y). 65:1680–1692.

Ashman, T. L. 1999. Determinants of sex allocation in a gynodioecious wild strawberry: Implications for the evolution of dioecy and sexual dimorphism. J. Evol. Biol. 12:648–661.

Ashman, T.-L., and C. J. Majetic. 2006. Genetic constraints on floral evolution: a review and evaluation of patterns. Heredity (Edinb). 96:343–52.

Bailey, M. F., L. F. Delph, and C. M. Lively. 2003. Modeling gynodioecy: novel scenarios for maintaining polymorphism. Am. Nat. 161:762–776.

Barrio, M., and A. L. Teixido. 2015. Sex-dependent selection on flower size in a large-flowered Mediterranean species: an experimental approach with Cistus ladanifer. Plant Syst. Evol. 301:113–124.

Caruso, C. M., and a. L. Case. 2007. Sex ratio variation in gynodioecious Lobelia siphilitica: Effects of population size and geographic location. J. Evol. Biol. 20:1396–1405.

Chang, S.-M. 2006. Female compensation through the quantity and quality of progeny in a gynodioecious plant, Geranium maculatum (Geraniaceae). Am. J. Bot. 93:263–270.

Chang, S.-M. 2007. Gender-specific inbreeding depression in a gynodioecious plant, Geranium maculatum (Geraniaceae). Am. J. Bot. 94:1193–1204.

Charlesworth, D., and B. Charlesworth. 1978. A model for the evolution of dioecy and gynodioecy. 112:975–997.

Charlesworth, D., and B. Charlesworth. 1979. The evolutionary genetics of sexual systems in flowering plants. 205:513–530.

Charlesworth, D., and F. R. Ganders. 1979. The population genetics with cytoplasmic-genic male-sterility. Heredity (Edinb). 43:213–218.

Dorken, M. E., and E. T. a Mitchard. 2008. Phenotypic plasticity of hermaphrodite sex allocation promotes the evolution of separate sexes: an experimental test of the sex-differential plasticity hypothesis using Sagittaria latifolia (Alismataceae). Evolution 62:971–8.

Dufay, M., and E. Billard. 2012. How much better are females? the occurrence of female advantage, its proximal causes and its variation within and among gynodioecious species. Ann. Bot. 109:505–519.

Dufay, M., J. Cuguen, J.-F. Arnaud, and P. Touzet. 2009. Sex ratio variation among gynodioecious populations of sea beet: can it be explained by negative frequency-dependent

selection? Evolution 63:1483–97.

Emery, S. N., and D. E. McCauley. 2002. Consequences of inbreeding for offspring fitness and gender in Silene vulgaris, a gynodioecious plant. J. Evol. Biol. 15:1057–1066.

Gouyon, P., Vichot, F., Van Damme, J. M. M. 1991. Nuclear-cytoplasmic male sterility: single point equilibria versus limit cycles. Am. Nat. 137:498–514.

Koelewijn, H. P., and J. M. M. van Damme. 2005. Effects of seed size, inbreeding and maternal sex on offspring fitness in gynodioecious Plantago coronopus. J. Ecol. 93:373–383.

Landergott, U., J. J. Schneller, R. Holderegger, and J. D. Thompson. 2009. Sex-ratio variation and spatial distribution of nuclear and cytoplasmic sex-determining genes in gynodioecious Thymus praecox across altitudinal gradients. 23–42.

Lloyd, D. G. 1975. The maintenance of gynodioecy and androdioecy in angiosperms. Genetica 45:325–339.

Lloyd, D. G. 1974. Theoretical sex ratios of dioecious and gynodioecious angiosperms. Heredity (Edinb). 32:11–34.

McCauley, D. E., and M. F. Bailey. 2009. Recent advances in the study of gynodioecy: the interface of theory and empiricism. Ann. Bot. 104:611–20.

Mutikainen, P., and L. F. Delph. 1998. Inbreeding depression in Lobelia siphilitica: among-family differences override between-morph differences. Evolution (N. Y). 52:1572–1582.

Renner, S. S. 2014. The relative and absolute frequencies of angiosperm sexual systems: Dioecy, monoecy, gynodioecy, and an updated online database. Am. J. Bot. 101:1588–1596.

Spigler, R. B., and T.-L. Ashman. 2012. Gynodioecy to dioecy: are we there yet? Ann. Bot. 109:531–43.

Yu, Q., E. D. Ellen, M. J. Wade, and L. F. Delph. 2011. Genetic differences among populations in sexual dimorphism: Evidence for selection on males in a dioecious plant. J. Evol. Biol. 24:1120–1127.

CHAPTER FOUR

POLLEN DISPERSAL DISTANCES IN POPULATIONS OF THE GYNODIOECIOUS *GERANIUM MACULATUM* THAT VARY IN THEIR FEMALE FREQUENCY.[1]

[1]Christopher, Dorothy A. and S.-M. Chang. To be submitted to *Annals of Botany*.

Abstract

Gene flow within populations is an important determinant of population structure that can influence the composition of the local gene pool and the strength of selective pressures. In many understory herbaceous species, pollen dispersal is the principal mechanism of gene flow. Pollen dispersal can be influenced by many factors, including pollinator behavior and plant sexual system. Separate sexes changes the density of pollen-bearing individuals, which in turn affects the way in which pollen in moved throughout the population. We examined how pollen dispersal distances differ between populations of the gynodioecious *Geranium maculatum* that vary in their female frequency from 0-0.34. We found that pollen in high female frequency populations traveled a significantly shorter distance than pollen in hermaphrodite-only populations. Low and mid female frequency populations had intermediate pollen dispersal distances. In addition, populations showed spatial structuring of the sexes, in which females were clustered in small portions of the population. These results have implications for sexual system evolution in this species. Hermaphrodites experience a female frequency that is less than the population sex ratio, suggesting that the realized selective pressure exerted by females is reduced, making dioecy unlikely to evolve in this system.

Introduction

Plants rely on external agents, such as animals and abiotic vectors, to transfer their pollen between individuals for outcross mating to occur. The distance that pollen is carried defines the patterns of mating and seed parentage in a population (Silvertown and Charlesworth 2001). These mating patterns, in turn, impact the distribution of not only the alleles but also the resulting genotypes and phenotypes in a population, which can influence natural selection and the strength of selective pressures. It is therefore important to understand how gene flow and—if gene flow is limited—the genetic structure within a population can influence these mating patterns.

Pollen dispersal distances, particularly in insect pollinated species, are generally expected to be rather limited, which creates some challenges for the reproduction of such species. For example, the probability of mating between individuals in a population will decrease with increasing distance between them. As a result, even for a completely outcrossing species, the mixing of alleles within a population may not be entirely panmictic. In addition, depending on the density of flowering individuals and their distribution throughout the population, individuals may have uneven contributions of their pollen to the outcrossing pollen pools, leading to uneven genetic contributions to the next generation (Levin and Kerster 1974; Godoy and Jordano 2001; Burczyk et al. 2002; Wright and Meagher 2004). In populations where inter-plant distance is high or the overall plant density is low, limited pollen dispersal can also result in pollen limitation and reduce seed set for plants (e.g., de Cauwer et al. 2010). Finally, if mating and dispersal are highly localized, and there is high variance in individuals' reproductive success, genetic drift can be strong and uneven across the population. Pollen dispersal distances therefore have implications for inbreeding, effective population size, and population structure. In other

words, pollen dispersal strongly influences intra-population gene flow, highlighting the importance of understanding how pollen dispersal varies either within or among different populations.

Limited gene flow can cause within population genetic structure, which in turn defines the genetic neighborhood. The neighborhood determines the degree of genetic subdivision in a population as well as the composition of the local gene pool. Classic evolutionary theory has shown that, when selection is absent, the amount of genetic differentiation in a population depends mostly on the neighborhood size (Wright 1946, 1951). Therefore, to be able to estimate evolutionary processes that influence genetic variation and fitness, it is necessary to have an accurate estimate of neighborhood size and within population gene flow.

The neighborhood size is largely determined by the distance that pollen travels. While plants can disperse genetic material via pollen and seeds, pollen flow is the primary mechanism of gene flow because seed dispersal in understory species that have no external means of dispersal is often limited (Sork 2015). Additionally, the pollen flow to seed flow ratio is generally greater than 1, even in highly selfing species (Ennos 1994), thus pollen dispersal becomes the principal mechanism of gene flow. Pollen that is dispersed by an insect vector can be affected by many factors, including: pollen presentation, floral display, and pollen transfer efficiency, among others. All of these can affect pollination success and subsequently seed set and fitness.

An additional factor that may influence pollen dispersal is the plant sexual system. When sexual functions are separated into different individuals, the density of pollen-bearing individuals changes and, as a result, pollinator behavior may also change when visiting single sex plants (Fenster 1991). For example, the presence of females may influence pollinator behavior, as

female flowers generally experience reduced pollinator visitation relative to hermaphrodites (reviewed in Delph 1996). This can shape patterns of pollen flow within a population in which females are present (hereafter, dimorphic populations), thus the presence of females in a population can lead to a different pollen dispersal patterns from populations without females. For example, we might expect to see increased levels of gene flow when females are present because pollinators increase travel distances in search of hermaphrodite flowers. Alternatively, gene flow might be more limited in populations with females because pollinators may avoid females and hence remain in patches of pollen bearing (hermaphroditic or male) plants. Additionally, sexes tend to be spatially structured in populations (McCauley and Taylor 1997; McCauley and Bailey 2009; Sanderson et al. 2016). Thus, the impact of density and pollinator visitation on an individual may depend more on the sex ratio of their immediate neighborhood and not the population sex ratio. It is, therefore, important to consider the sex ratio, both at the population and neighborhood level, when estimating of pollen dispersal distance. Understanding how pollen dispersal patterns can influence plant fitness and selective pressures in species with separate sexes requires an estimate of the distance that pollen travels in the context of the population sex ratio.

Gynodioecy, in which separate female and hermaphrodite individuals occur in a species, is an appropriate model to use to investigate questions of pollen flow in populations with separate sexes. In particular, the gynodioecious species *Geranium maculatum* exhibits the characteristics described above: Female flowers are less attractive to pollinators and are visited at half the rate of hermaphrodite flowers (Van Etten and Chang 2014). The distribution of females is patchy; sexes are clustered, which can influence neighborhood structure because pollen clouds received by maternal plants are determined mainly by the local neighborhood (Oddou-Muratorio

et al. 2006). Finally, the sex ratio in *G. maculatum* varies from 0-35% female, which allows us to make comparisons between populations that have different female frequencies. Estimating pollen dispersal distances and neighborhood size is important to understanding the evolutionary trajectory of *G. maculatum* populations.

To elucidate how the sex ratio influences pollen flow within gynodioecious populations, we estimated the pollen dispersal distances in ten populations of the gynodioecious *Geranium maculatum*. We asked the following questions: 1) What is the average distance of pollen travel among populations of *G. maculatum*? 2) Are pollen dispersal distances different between populations with and without females? 3) What is the neighborhood size and does it vary depending on the female frequency? In the latter two questions, we expect because pollinators discriminate against female flowers, populations with females will show decreased pollen dispersal distances because pollinators will remain in smaller hermaphrodite patches.

Methods

Study System

Geranium maculatum L. (Geraniaceae) is a rhizomatous perennial herb that occurs from southern Canada to the southeastern US and west to the Great Plains. Plants flower in mid-spring, producing 6-10 flowers per inflorescence (Agren and Wilson 1991; Chang 2006). Flowers are generalist pollinated, mainly by small bees and flies. Hermaphrodites will produce full seed set when hand pollinated; however, because flowers are protandrous, selfing rates in natural populations are almost 0 (Van Etten and Chang 2014). Fruits mature approximately one month after pollination, producing up to 5 seeds per fruit. Seeds are dispersed up to 3m from the

maternal plant by dehiscence of the schizocarp (Stamp and Lucas 1983). Population female frequency ranges from 0-0.45.

Population sampling

In 2014, we located ten populations in northern Georgia and western North Carolina: three populations were hermaphrodite-only; two populations had low female frequency, 2-3%, two populations were 11-12% female; and three populations had high female frequency, 30-35% (Fig. 4.1 and Table 4.1). We obtained the sex ratio for each population by counting the number of female and hermaphrodite individuals flowering in each population. Females can be visually identified by their smaller flower size and the presence of aborted anthers that lack pollen. We counted the number of females and hermaphrodites for three years, 2014, 2015, and 2016, and the sex ratio remained consistent across years (see details in Results).

In each population, we mapped the location of every inflorescence and recorded the individual's sex. We laid two meter tapes perpendicular to each other along the outside edges of the population, and the distance of each inflorescence from the meter tapes was recorded to obtain (x,y) coordinates for each inflorescence. Because of *G. maculatum*'s rhizomatous growth form, genets have an inflorescence that grows from one end of the rhizome, and there may be vegetative shoots that grow from the other end. In recording only inflorescences, we ensured we were not resampling genets. From each inflorescence stalk (maternal plant), we collected leaf tissue (preserved in silica gel until DNA extraction) and 10 seeds per inflorescence.

DNA extraction and genotyping

In each population, we genotyped all maternal individuals and seeds at eight microsatellite loci. DNA for maternal plants was extracted from dried leaf tissue. Seeds were first prepared in the following way before DNA extraction: scarified seeds were soaked for 1 week at (4°C) to soften the seed coat (maternal tissue) which was carefully removed before DNA extraction using the remaining seed tissue.

We extracted DNA using a modified CTAB protocol (Doyle and Doyle 1990). Microsatellite fragments were amplified using a 3-primer touchdown PCR program under the following conditions: 10 minutes of denaturing at 95°C; followed by 10 cycles of denaturing at 95°C for 30 seconds, annealing at 65°C for 30 seconds, decreasing by 1°C every cycle, extension for 30 seconds at 72°C. This was followed by 30 cycles of denaturing at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension for 30 seconds at 72°C. Lastly, there was a final extension for 20 minutes at 72°C. PCR products were sized using an internal dye-labeled size standard (ROX500, UGA Georgia Genomics Facility) and analyzed on an ABI 3730 capillary sequencer. Allele calls were made using PeakScanner (ABI v1.0).

Data analyses—

Spatial structure

To determine if sexes were aggregated within dimorphic populations, we used a test of spatial autocorrelation. The autocorrelation coefficient *r* is bounded by 1 and -1. Positive values indicate positive spatial autocorrelation, that plants of the same sex are clustered. Negative values indicate that plants of the opposite sex are clustered within a population. 95% confidence intervals were generated with a permutation test. Spatial positions were permuted 1000 times to

generate a distribution of values under the null hypothesis of no spatial correlation. We performed these tests using the *adegenet* package in R v.3.3.2 (R Core Development Team, 2017).

Mating system

We estimated selfing and biparental inbreeding rates in all populations with the following mating system parameters: the multilocus outcrossing rate (t_m), multilocus selfing rate (s_m=1-t_m); and the mean single locus selfing rate (t_s). Using the latter two estimates, we calculated the biparental inbreeding rate as $b=t_m-t_s$. The selfing rate for females should be 0, however a non-zero value is possible if the diversity in the genetic markers is not sufficient to assign parentage correctly or to distinguish selfing from mating with relatives. For each parameter we generated 95% confidence intervals with 5000 bootstrap iterations with maternal family as the resampling unit. This allowed us to determine if selfing and inbreeding rates were significantly different from 0. Mating system analyses were performed in MLTR v.3.2 (Ritland 2012).

We used a generalized linear mixed model to compare whether mating system parameters were significantly different between hermaphrodite-only and dimorphic populations and between the sexes within a population using the *lme4* package (Pinheiro et al. 2013) in R v.3.3.3 (R Core Development Team 2017). Population was coded as a random effect; population type (hermaphrodite-only or dimorphic) was a fixed effect. Since we had several populations with very few females, we also analyzed whether mating system parameters were different between high female frequency populations and all others, including hermaphrodite-only and low female frequency populations grouped together.

Pollen dispersal distances

The eight microsatellite loci used have an average of 8 alleles per locus, which yields an exclusion probability of 98% (calculated in GenAlEx (Peakall and Smouse 2012)), indicating sufficient power for calculating pollen dispersal parameters.

Using the mapped mother-offspring genotypic data, we estimated contemporary pollen dispersal using the KINDIST method implemented in POLDISP (Robledo-Arnuncio et al. 2007). We prepared the data for each population in the following way: First, no mismatches between mother and offspring; if a mismatch was identified, we re-genotyped that locus in both individuals. If the mismatch persisted, that allele was transformed to missing data in the offspring. Second, there was no missing data for mothers, and seeds that were the result of selfing were omitted. We used CERVUS (Kalinowski et al. 2007) to identify potential selfed seeds. We used KINDIST to calculate the correlated paternity among maternal families (whether offspring from different maternal families share a father), and subsequently calculated whether correlated paternity was inversely correlated with distance between maternal plants, an assumption that must be met to estimate pollen dispersal distances in KINDIST, and was satisfied in all populations, validating the use of KINDIST for subsequent analyses. We first estimated dispersal parameters for each population separately and then averaged within hermaphrodite-only and dimorphic populations. We tested whether parameters were significantly different between population type with an ANOVA. We tested whether the pollen dispersal distances were due to the density of individuals in the populations. We regressed the population density on the pollen dispersal distances, then took the residuals from that model and regressed those against the population female frequency. These residuals should have the effect of density removed; if a

correlation is still present between the female frequency and the residuals, this indicates that density is not the driver of the pollen dispersal distances.

Results

Spatial structure

All dimorphic populations except LB show spatial structuring of the sexes (Fig. 4.2). Spatial autocorrelation was the strongest in EL, where sexes were autocorrelated within the first three distance classes, up to 0.6m. OT, SR2, SU, and TE showed spatial structuring within the first distance class, up to 0.2m. BH sexes were autocorrelated within the first two distance classes, up to 0.4m.

Mating system

Both sexes were almost completely outcrossing in all populations (multilocus outcrossing rate = 0.90-1.01, Table 4.2). Using the difference between single locus (t_s) and multilocus outcrossing rates (t_m), biparental inbreeding rates (t_m-t_s) were estimated to be low, ranging from -0.01-0.09. In two out of four dimorphic populations, females had slightly higher biparental inbreeding values than hermaphrodites but the difference is minimal (Table 4.2). Mating system parameters for all populations are given in Table 4.2.

Pollen dispersal

Pollen dispersal distances were significantly lower in dimorphic populations (Table 4.3, $F_{1,9}$=6.3, p=0.015). The average dispersal distance of hermaphrodite-only populations was 1.70 m (SE 0.06), while in dimorphic populations the average distance was 1.43 m (SE 0.08). The

values in the low female frequency populations were more similar to the hermaphrodite-only populations. The average dispersal distance in only the HFF populations was 1.20 m (SE 0.05).

The density of adults in populations varied from 1.9-3.05 (Table 4.3); there was a trend in which adult density in hermaphrodite-only populations was less than in dimorphic populations ($F_{1,9}$=5.17, p=0.052). The proportion of individuals that participated in pollination ranged from 0.31-0.74 (Table 4.2). A significantly lower proportion of individuals in dimorphic populations participated in pollination (Table 4.2, $F_{1,9}$=11.06, p=0.020). The average within sibship correlated paternity was not different between population types (Table 4.3, $F_{1,9}$=1.87, p=0.29), however the among sibship correlated paternity was significantly lower in hermaphrodite-only populations (Fig. 4.3 and Table 4.3, $F_{1,9}$=20.01=, p<0.01). A regression between the residuals of a model regressing density and dispersal distances and female frequency showed a correlation between the residuals and female frequency (Fig. 4.4, R^2=-0.63, p<0.001).

Discussion

Our study showed that pollen dispersal distances vary depending on the female frequency in the population. In hermaphrodite-only populations, the average distance of pollen travel was 1.7 m, and the average distance in populations with females was 1.4 m; pollen on average was dispersed farther in hermaphrodite-only populations. This difference increased when compared to only the high female frequency populations (SU, SR2, and TE). In these populations the pollen dispersal distance was 1.2 m. In the low female frequency populations (EL and LB), the patterns were more similar to the hermaphrodite-only populations. These populations were only 2% female, which was only 2-4 female individuals. It makes sense that this low number of female individuals by itself would not exert a strong influence on the population dynamics and

gene flow; these populations, indeed, tend to behave similarly to those without any females. The populations with a moderate female frequency (BH, 11% female, and OT, 12% female), had pollen dispersal distances that were intermediate between hermaphrodite-only and high female frequency populations. Taken together, our results suggest female frequency in a population affected how pollen was moved in the population, specifically that as female frequency increased, pollen dispersal distances decreased. We discuss several potential mechanisms for this pattern below.

First, the density of hermaphrodites may be an important factor. The number and density of hermaphrodites in hermaphrodite-only populations is greater. This can influence pollinator behavior, which in turn may influence dispersal distances (Van Etten and Chang 2014). *G. maculatum* is almost entirely outcrossed, which means that it is dependent on pollinators for pollination and fertilization; thus, how pollinators move pollen throughout a population is extremely important for seed set and fitness. When pollen movement is restricted, a common result is pollen limitation, in which individuals do not receive a full pollen load and seed set decreases. In the gynodioecious species *Glechoma hederecea*, seed set of females decreased with increasing distance to the nearest pollen source, suggesting that the bumblebee pollinators did not successfully transfer pollen long distances (Widén and Widén 1990). Thus, restricted gene flow may have negative consequences for individual fitness, particularly for females, in a population. In the *G. maculatum* populations in this study, pollen dispersal distances were lowest in high female frequency populations, which suggests females in these populations might experience pollen limitation. In fact, we do see a pattern of higher seed set in hermaphrodite-only populations (chapter 2). Individuals in these populations produce an average of 4.8 (SE 0.5),

while individuals in dimorphic populations produce an average of 3.4 (SE 0.8) seeds, suggesting pollen limitation may be occurring in dimorphic populations.

It is not only the distances that pollinators travel that may reduce seed set in females of *G. maculatum*, but also pollinator behavior. In a previous study, Van Etten and Chang (2014) showed that pollinators discriminate against females in *G. maculatum* populations, and females are visited at half the rate of hermaphrodites, which results in reduced pollen deposition and seed set. This, combined with decreased pollen dispersal when females are present, suggests females may be at a substantial fitness disadvantage relative to hermaphrodites, which may have implications for their long-term persistence in populations (discussed more below).

Our study also found that populations showed significant spatial structuring of the sexes; sexes are more likely to be found clustered with individuals of their same sex than next to individuals of the opposite sex. This results in a patchy distribution of the sexes across the population. This is similar to patterns seen in the gynodioecious *Silene vulgaris*, in which the patchy distribution of the sexes has the potential influence the social context experienced by individuals, thereby shaping the strength of selection on individuals (Sanderson et al. 2016). The population structure can also influence pollen performance. Another study also in *S. vulgaris*, found that neighboring individuals were likely to be related to each other, and that pollen performance (i.e., fertilization success) increased with increasing distance between paternal and maternal plants (Glaettli et al. 2006). This was probably due to increased inbreeding at close distances. Limited pollen flow also reduces male fecundity in the gynodioecious *Beta vulgaris* (De Cauwer et al. 2012).

In *G. maculatum*, we also see significant spatial genetic structure within populations (Van Etten et al. 2014). Thus we might expect that siring success would increase when pollen was

transferred to a maternal plant that was a farther distance away. However, with the decreased pollen dispersal distances in high female frequency populations, pollen may not be transferred longer distances to unrelated individuals, which increases the opportunities for inbreeding. While rates of biparental inbreeding overall were low, the highest rates were found in females of populations OT, SU, and TE, which are middle and high female frequency populations. This pattern of biparental inbreeding is similar to what Van Etten et al. (2014) found, in which females in certain populations had higher rates of biparental inbreeding. These results together suggest that reduced pollen dispersal distances result in increased mating between relatives, and because pollinators discriminate against females, females receive less pollen from unrelated pollen pools. This may result in lower female fitness over time.

Implications for the maintenance of gynodioecy

The evolution of gynodioecious populations is predicted to move in one of three ways: remain stable in gynodioecy, revert to hermaphroditism, or evolve dioecy. For dioecy to evolve, there are several steps that must occur. The increased availability of ovules from females puts selective pressure on hermaphrodites to increase pollen production at the expense of seed production. Hermaphrodites are predicted to become "more male" as female frequency rises, which facilitates the establishment of a female sterility mutation (Bailey et al. 2003; Spigler and Ashman 2012).

We might then predict that in HFF populations of *G.* maculatum, hermaphrodites should experience this selective pressure caused by the increased number of females, and subsequently allocate more to male function at the expense of female function. However, we did not find this pattern (chapter 2), and the population gene flow patterns revealed in this study may be the

reason. We found that in HFF populations, pollen dispersal distances are reduced and the sexes are spatially clustered. This means that the sex ratio that a hermaphrodite experiences may not be the population sex ratio, but rather the neighborhood sex ratio, defined as a circle around the focal plant with radius equal to the pollen dispersal distance (Wright 1951; Silvertown and Charlesworth 2001). In the HFF populations, the average female frequency of the neighborhood that surrounds hermaphrodites is 0.12 (SE 0.16). This is considerably less than the population sex ratio, and therefore makes it unlikely that hermaphrodites would experience strong selective pressure to increase male function.

Finally, we found within population gene flow is reduced in high female frequency populations. This potentially has implications for the spread of male sterility alleles. If mating occurs mainly between individuals that are near each other in the population, this may limit the movement of male sterility alleles throughout the population. In many gynodioecious species, including *Lobelia siphilitica* (Caruso and Case 2007), *Beta vulgaris* (Dufay et al. 2009), *Thymus vulgaris* (Landergott et al. 2009), and others, female frequency can reach as high as 90%. In *G. maculatum*, the highest female frequency we observe is 35-40%. It may be that reduced gene flow within populations with middle and high female frequencies prevents the spread of male sterility alleles, because individuals with male sterility alleles are mating with nearby individuals that already posses male sterility alleles, and are not mating with individuals in other areas of the population, which would introduce male sterility alleles to those individuals. Furthermore, female compensation in seed production is minimal (chapter 2). Under nuclear control of male sterility, females must produce twice the number of seeds as hermaphrodites. We found females produce only 1.2 times as many seeds, which may it difficult for females to increase in frequency in a population. Taken together, these results suggest dioecy is unlikely to evolve in this system.

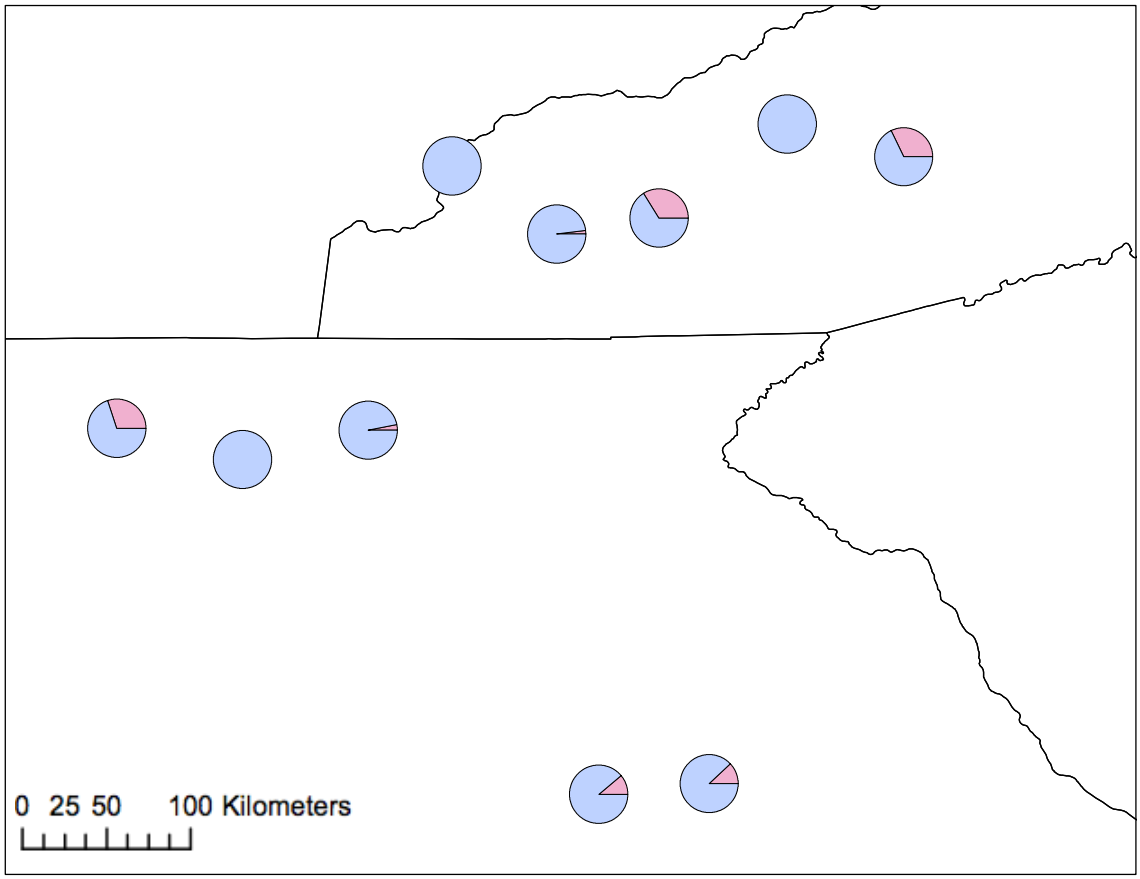


Fig. 4.1. Locations of sampled populations. Blue and pink indicate the proportion of the population hermaphrodite and female, respectively.

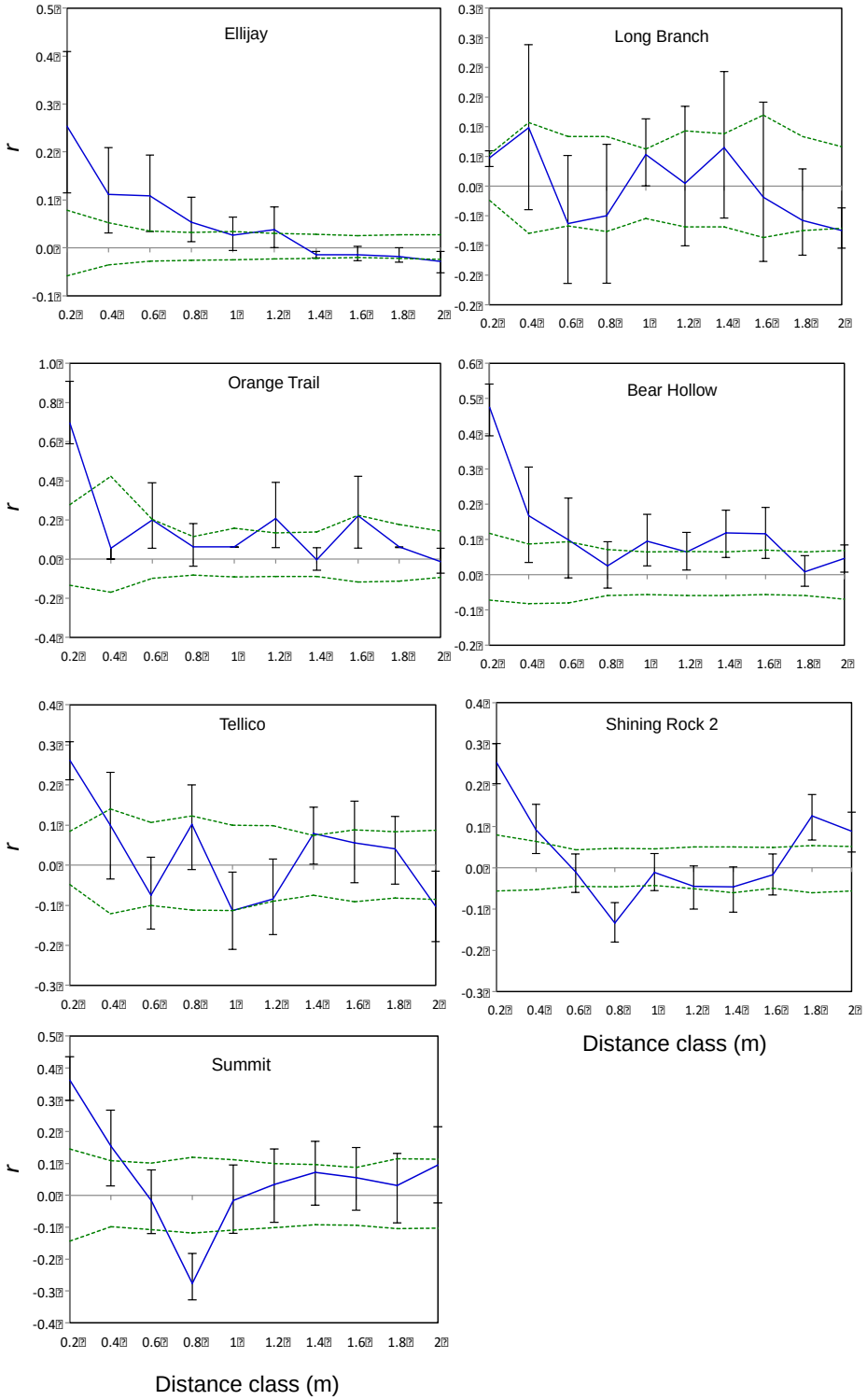


Fig. 4.2. Spatial autocorrelograms for all dimorphic populations; EL, 2% female; LB, 3%; OT, 12%; BH, 12%; TE, 34%; SR2, 32%; SU, 30%.

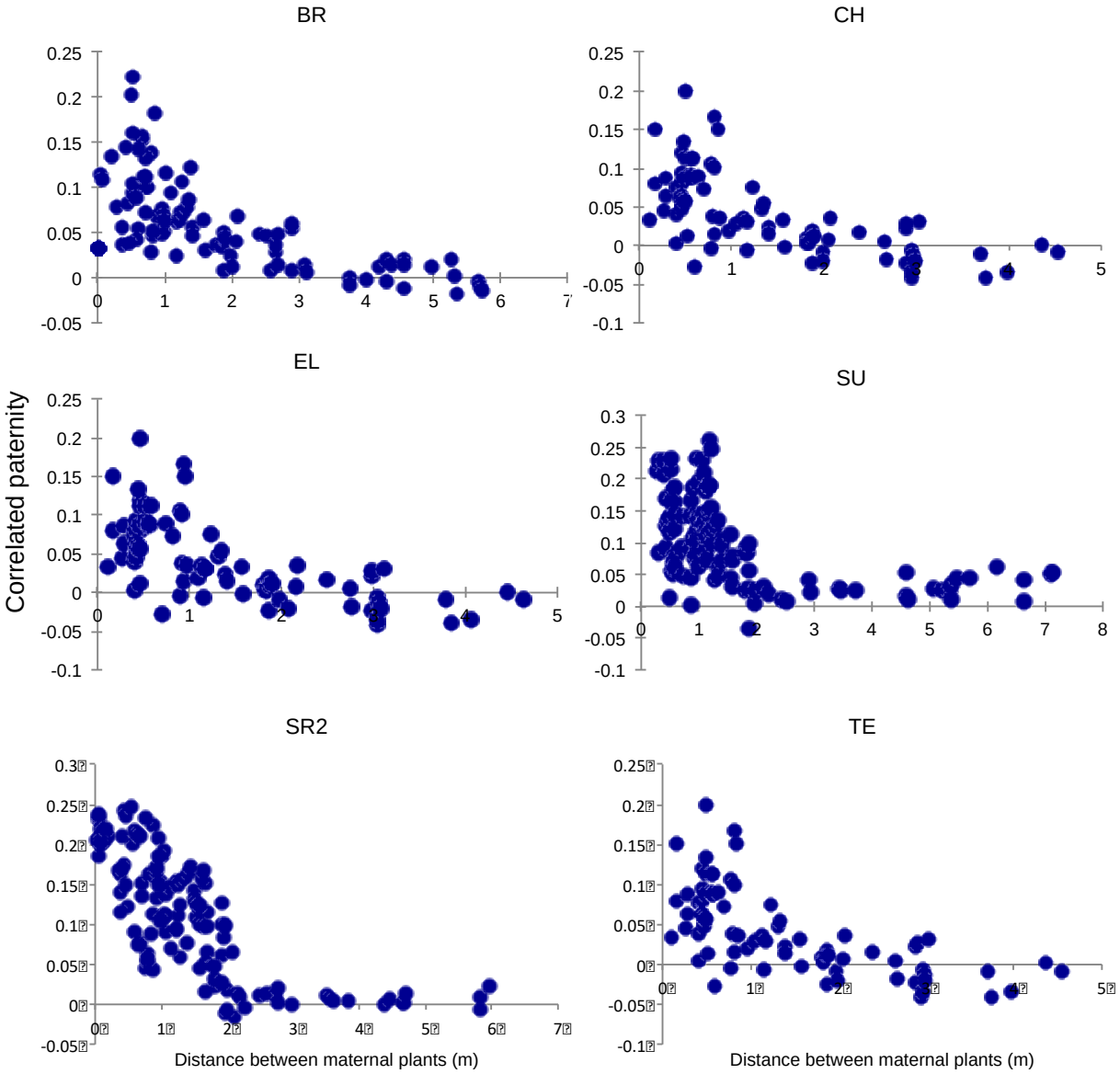


Fig. 4.3. Among maternal sibship correlated paternity for all populations. Each point represents a pair of progeny arrays (comparison of relatedness between progeny arrays of two maternal plants). Maximum value possible is 0.25, which indicates half-sibs, i.e., offspring share a father. Population percent female: BR, 0%; CH, 0%; EL, 2%; SU, 30%; SR2, 32%; TE, 34%; BH, 11%; LB, 2%; OT, 12%.

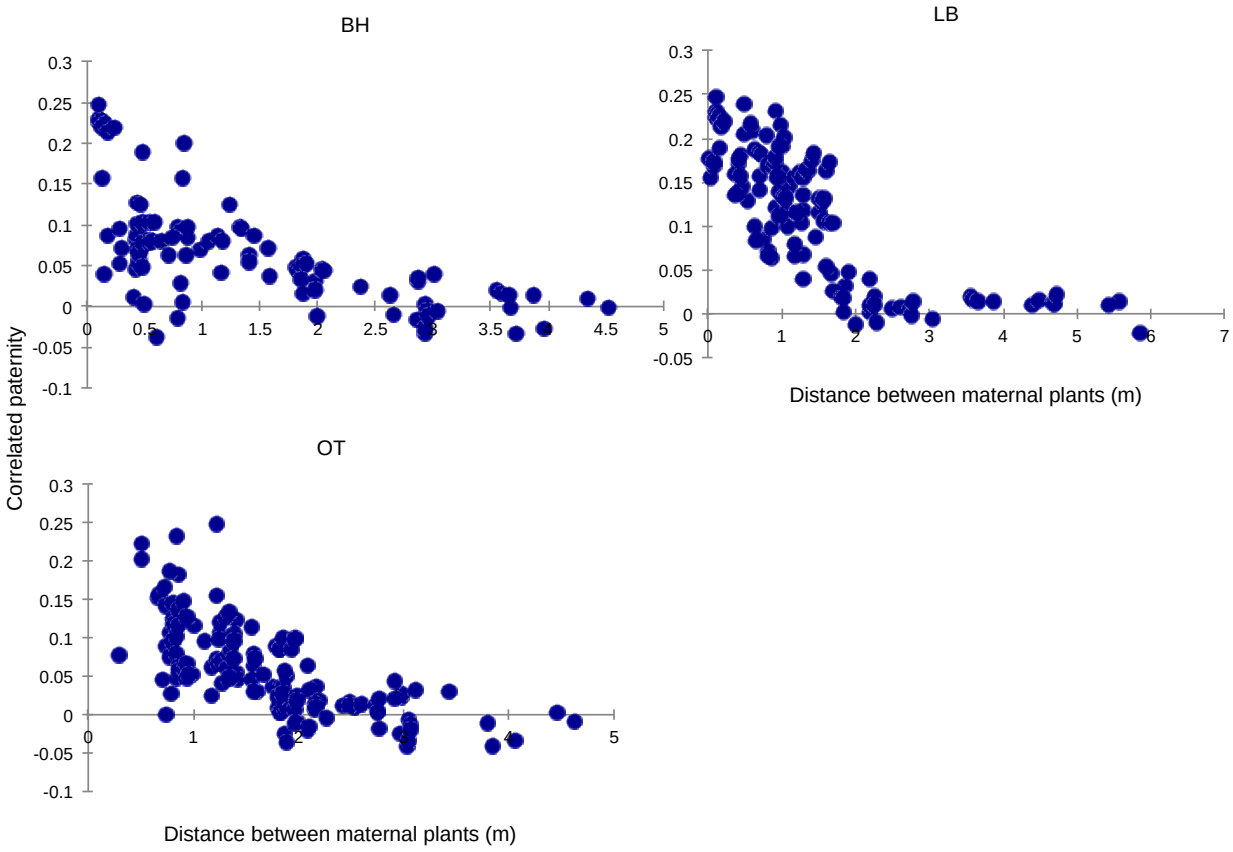


Fig 3 continued. Among maternal sibship correlated paternity for all populations.

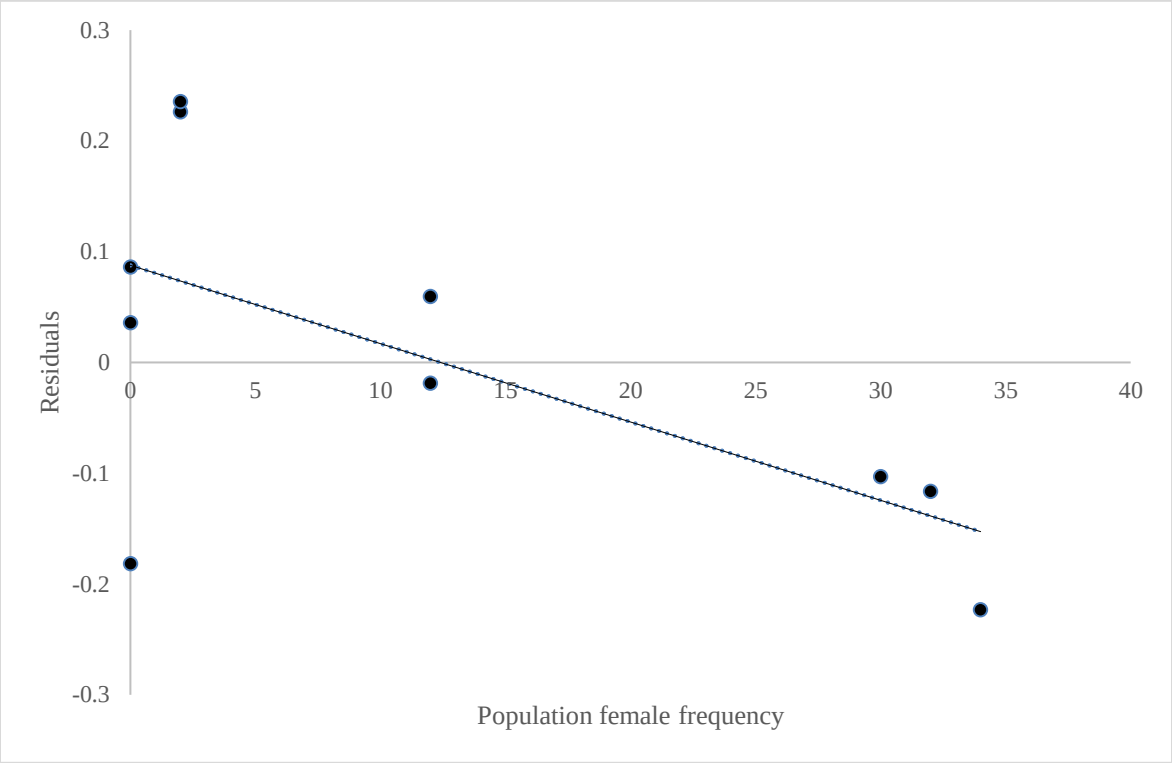


Fig. 4.4. Plot of the residuals from a model regressing population density and pollen dispersal distance and population female frequency.

Table 4.1. Populations sampled in this study, including percent female (%F) in the three years of the study, 2014, 2015, and 2016.

Population	*# Individuals*	*% F 2014*	*%F 2015*	*%F 2016*	*GPS coordinates*
Turtle Pond (TP)	55	0	0	0	34.77426, -84.66132
Cherohala (CH)	62	0	0	0	35.32673, -83.86663
Blue Ridge (BR)	42	0	0	0	35.44194, -83.08073
Ellijay (EL)	57	2	2	2	34.770084, -84.5899
Long Branch (LB)	105	2	3	3	35.23798, -83.626233
Bear Hollow (BH)	147	11	12	12	33.92672, -83.386391
Orange Trail (OT)	152	12	14	12	33.90280, -83.381355
Summit (SU)	74	30	30	30	34.77438, -84.67049
Shining Rock 2 (SR2)	82	32	32	32	35.422394, -82.92228
Tellico (TE)	64	34	34	34	35.27592, -83.50613

Table 4.2. Summary of mating system parameters and estimates of inbreeding depression for all populations.

Pop.	*Sex*	t_m	t_s	t_m-t_s	*Fixation index (F)*
Hermaphrodite-only populations					
TP					
	Hermaphrodites	0.98	0.98	0.00	0.05
CH					
	Hermaphrodites	0.97	0.96	0.01	0.02
BR					
	Hermaphrodites	0.99	0.99	0.00	0.05
Dimorphic populations					
EL					
	Females	0.99	0.98	0.01	0.02
	Hermaphrodites	0.98	0.97	0.01	0.01
LB					
	Females	0.99	0.98	0.01	0.01
	Hermaphrodites	0.99	0.97	0.02	0.02
BH					
	Females	0.98	0.98	0.00	0.01
	Hermaphrodites	0.98	0.97	0.01	0.01
OT					
	Females	0.98	0.96	0.03	0.01
	Hermaphrodites	0.99	0.97	0.02	0.01
SU					
	Females	0.98	0.90	0.08	0.05
	Hermaphrodites	1.01	0.98	0.03	0.06
SR2					
	Females	0.98	0.96	0.02	0.04
	Hermaphrodites	0.96	0.97	-0.01	0.01
TE					
	Females	0.99	0.90	0.09	0.08
	Hermaphrodites	0.98	0.96	0.02	0.02

Table 4.3. Parameters of pollen dispersal for a) all populations, and b) mean values (SE) for hermaphrodite-only and dimorphic populations. Values that are significantly different at the $p<0.05$ level are in bold. Dimorphic populations (all) and HFF populations are compared to the hermaphrodite-only populations. Abbreviations: *D*, density of adults per square meter; D_{Ep}/D, proportion of individuals that participate in pollination; r_p, average within sibship correlated paternity; r_{Ap}, average among sibship correlated paternity; N_{EP}, number of effective pollen donors; HFF, includes high female frequency populations only (SU, SR2, and TE).

a)

Pop.	*% female*	*D*	D_{Ep}/D	N_{EP}	r_p	r_{Ap}	*pollen dispersal (m)*
TP	0	1.90	0.65	3.68	0.45	0.18	1.84
CH	0	2.24	0.70	4.75	0.42	0.20	1.70
BR	0	2.03	0.74	5.12	0.40	0.15	1.55
EL	2	2.58	0.65	5.25	0.50	0.17	1.65
LB	2	2.65	0.68	5.05	0.48	0.20	1.62
BH	12	2.41	0.45	4.60	0.43	0.21	1.50
OT	12	2.30	0.58	3.71	0.48	0.19	1.64
SU	30	3.01	0.33	2.95	0.50	0.24	1.08
SR2	32	2.45	0.35	3.05	0.49	0.22	1.38
TE	34	2.67	0.31	3.22	0.48	0.22	1.15

b)

Pop. type	*D*	D_{Ep}/D	r_p	r_{Ap}	*pollen dispersal (m)*
H-only	**2.05(0.08)**	**0.70(0.02)**	**0.42(0.01)**	**0.18(0.01)**	**1.70(0.06)**
Dimorphic (all)	**2.58(0.08)**	**0.48(0.05)**	**0.48(0.008)**	**0.21(.008)**	**1.43(0.08)**
Dimorphic (HFF)	**2.71(0.09)**	**0.33(0.006)**	**0.49(0.003)**	**0.23(0.003)**	**1.20(0.05)**

References

Bailey, M. F., L. F. Delph, and C. M. Lively. 2003. Modeling gynodioecy: novel scenarios for maintaining polymorphism. Am. Nat. 161:762–776.

Caruso, C. M., and a L. Case. 2007. Sex ratio variation in gynodioecious Lobelia siphilitica: effects of population size and geographic location. J. Evol. Biol. 20:1396–405.

Cauwer, I. D. E., J. Arnaud, E. Schmitt, and M. Dufay. 2010. Pollen limitation of female reproductive success at fine spatial scale in a gynodioecious and wind-pollinated species , Beta vulgaris ssp . maritima. 23:2636–2647.

Chang, S.-M. 2006. Female compensation through the quantity and quality of progeny in a gynodioecious plant, Geranium maculatum (Geraniaceae). Am. J. Bot. 93:263–270.

De Cauwer, I., J. F. Arnaud, E. K. Klein, and M. Dufay. 2012. Disentangling the causes of heterogeneity in male fecundity in gynodioecious Beta vulgaris ssp. maritima. New Phytol. 195:676–687.

Dufay, M., J. Cuguen, J.-F. Arnaud, and P. Touzet. 2009. Sex ratio variation among gynodioecious populations of sea beet: can it be explained by negative frequency-dependent selection? Evolution 63:1483–97.

Etten, M. L. Van, A. C. Deen, J. L. Hamrick, and S. Chang. 2014. Mating system contributes only slightly to female maintenance in gynodioecious Geranium maculatum (Geraniaceae). 113:464–470.

Glaettli, M., L. Pescatore, and J. Goudet. 2006. Proximity-dependent Pollen Performance in Silene vulgaris. Ann. Bot. 431–437.

JJ, D., and J. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus (Madison). 12:13–15.
Kalinowski, S. T., M. L. Taper, and T. C. Marshall. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Mol. Ecol. 16:1099–106.

Landergott, U., J. J. Schneller, R. Holderegger, and J. D. Thompson. 2009. Sex-ratio variation and spatial distribution of nuclear and cytoplasmic sex-determining genes in gynodioecious Thymus praecox across altitudinal gradients. 23–42.

McCauley, D. E., and M. F. Bailey. 2009. Recent advances in the study of gynodioecy: the interface of theory and empiricism. Ann. Bot. 104:611–20.

McCauley, D. E., and D. R. Taylor. 1997. Local population structure and sex ratio: Evolution in gynodioecious plants. Am. Nat. 150:406–419.

Oddou-Muratorio, S., E. K. Klein, and F. Austerlitz. 2006. Real-Time Patterns of Pollen Flow in

the Wild-Service Tree, Sorbus torminalis (Rosaceae). III . Mating Patterns and the Ecological Maternal Neighborhood. Molecular Ecology 93:1650–1659.

Peakall, R., and P. E. Smouse. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research--an update. Bioinformatics 28:2537–9.

Robledo-Arnuncio, J. J., F. Austerlitz, and P. E. Smouse. 2007. POLDISP: a software package for indirect estimation of contemporary pollen dispersal. Mol. Ecol. Notes 7:763–766.

Sanderson, B. J., M. E. Augat, D. R. Taylor, and E. D. B. Iii. 2016. Scale dependence of sex ratio in wild plant populations : implications for social selection. 1411–1419.

Sork, V. L. 2015. Gene flow and natural selection shape spatial patterns of genes in tree populations : implications for evolutionary processes and applications.

Spigler, R. B., and T.-L. Ashman. 2012. Gynodioecy to dioecy: are we there yet? Ann. Bot. 109:531–43.

Van Etten, M. L., and S. M. Chang. 2014. Frequency-dependent pollinator discrimination acts against female plants in the gynodioecious Geranium maculatum. Ann. Bot. 114:1769–1778.

Widén, B., and M. Widén. 1990. Pollen Limitation and Distance-Dependent Fecundity in Females of the Clonal gynodioecious herb Glechoma hederacea (Lamiaceae). Oecologia 83:191–196.

CHAPTER FIVE

PROGENY ARRAY SEX RATIOS FROM WITHIN AND AMONG POPULATION CROSSES OF *GERANIUM* MACULATUM.[1]

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Abstract

Gynodioecy is a sexual system in which both hermaphrodite and female sexes occur in a species. Females have been shown to arise via a male sterility mutation that causes anther development in hermaphrodites to be arrested. Understanding the genetic control of male sterility is important because it allows us to make predictions about the evolutionary stability of gynodioecy, the equilibrium population female frequency, and the strength of selection on reproductive traits. Previous work in *Geranium maculatum* has showed the genetic control of sex to be complex, and therefore in this study we took a quantitative genetic approach, treating male sterility as the quantitative trait. We completed crosses within and between populations, germinated the resulting seedlings and scored the sex of the progeny. We found a significant effect of the paternal population of origin as well as a positive correlation between the female frequency of the paternal population and the female frequency in the progeny arrays.

Introduction

Gynodioecy is a sexual system in which separate female and hermaphrodite sexes occur in a species. Gynodioecy is thought to evolve from a hermaphroditic ancestor (Richards 1997), and females have been shown to result from a male sterility mutation in a hermaphrodite that causes a plant to retain only female reproductive function (Frank 1989). Hermaphrodites in gynodioecious species are often self-compatible, and often there is a positive relationship between the selfing rate and the female frequency (Sun and Ganders 1986); it is therefore posited that females evolve and are maintained because they do not suffer from inbreeding depression to the same degree as hermaphrodites, thereby increasing their fitness in a population (Barrett 2002). Femaleness is therefore a trait that confers a selective advantage to those individuals in a population.

Identifying the genetic factors that control male sterility has long been a goal of researchers studying gynodioecious species; male sterility can be under either nuclear or cyto-nuclear control. Understanding the genetic control is important because it allows us to predict equilibrium population sex ratios as well as understand population dynamics such as the strength of selection on reproductive traits (Ashman and Majetic 2006; Barrett 2013). Conventional approaches have used reciprocal crosses between hermaphrodites, often with a known genetic background (i.e., whether it carries a male sterility allele and corresponding restorer of fertility). Progeny sex ratios can then be compared between individual crosses to understand the genetic control and potentially the number of loci involved (e.g., Belhassen 1991; Dudle et al. 2001; Weller et al. 2014).

In the gynodioecious species *Geranium maculatum*, a previous crossing experiment has shown the genetic control of male sterility to be complex (Van Etten 2009). This study showed

that there is most likely more than one nuclear locus involved. Van Etten found that when the distance between the parental populations of origin increased, the frequency of an intermediate phenotype increased, while the hermaphrodite frequency decreased. Additionally, *G. maculatum* populations show genetic structure between high female frequency populations and others (Chapter 1). With among population crosses, we can examine the effect of geographic distance as well as genetic distance between parental populations.

When a trait is complex, as it seems to be in *G. maculatum*, a useful approach is to use a quantitative genetic approach. While with this framework we will not get to exact type of genetic control, we can estimate the importance of maternal and paternal factors, including population of origin. This approach has been used in a previous study of male sterility (Taylor et al. 2001). They were able to estimate the effects of sire, dam, and their interaction, as well as examine these effects with crosses completed among populations. In this study, we used within population and well as among population crosses to examine how maternal and paternal factors, including their populations of origin, contribute to variation in progeny array sex ratios.

Methods

Study system

Geranium maculatum is a gynodioecious rhizomatous perennial herb. The species occurs from southern Ontario to Georgia and west to the Great Plains (Stamp and Lucas 1983). Population female frequency ranges from 0-0.35, with an average of 0.11 (Chapter 1). Females have aborted anthers and smaller flowers relative to hermaphrodites (Agren and Willson 1991). Hermaphrodites are self-compatible, although selfing rates in natural populations are low (<0.12)

(Van Etten et al. 2014), most likely due to protandry. Inbreeding depression is high, the cumulative post-dispersal inbreeding rate ranges from 0.38-0.84 (Chang 2007).

Populations

Individuals used in this crossing experiment were collected from populations located from Georgia north to New York, in 2014. We used the same populations for both the within and among population crosses; one population with low female frequency (R28A, 8% female), one population with an intermediate female frequency (TSF, 18% female), and three populations with high female frequency (BCSP, 28% female; SR2, 32% female; SU, 34% female) (Table 5.1).

Within population crosses

In each of the 5 populations, we randomly chose 4 hermaphrodites and 3 females. The individuals were crossed using a factorial crossing scheme such that each hermaphrodite was crossed with each female to generate 12 progeny arrays within each population (Table 5.2). We pollinated three flowers per cross to generate 15 seeds. *G. maculatum* has a germination rate of approximately 50%; in producing 15 seeds per cross we ensured we will have sufficient progeny germinating to score the sex.

The sex ratio of the progeny array was analyzed with a regression. All crosses (that is, all 16 progeny arrays from all 5 populations) were analyzed together, with sire and dam as fixed effects and population as a random effect. Because both sire and dam contribute to the nuclear variation while only dam contributes to the cytoplasmic variation, we estimated the portions of variation attributable to sire and dam as well as their interaction to interpret the nuclear and cytoplasmic effects between the two. Significant sire effects indicate that nuclear genotypes have

a significant effect on the progeny sex ratio. Significant dam effects, the maternal contribution to the progeny sex ratio, indicate that maternal effects, including nuclear, cytoplasmic and environmental, are important. Because all plants were grown in similar environmental conditions, we assume that maternal environmental effects are minimal. Significantly higher contribution by dam than sire would suggest that cytoplasmic genetic effects are significant for progeny sex ratios. A significant interaction between sire and dam would suggest that the specific pairing of the parents contributes to progeny sex ratios.

Among population crosses

For this set of crosses, we randomly chose 4 females and 4 hermaphrodites from each population from the same set of populations used for the within population crosses. Among population crosses were carried out such that each hermaphrodite pollinated the 4 females from the other four populations. We again pollinated three flowers per cross to generate 15 seeds. We pooled all seeds from these three flowers and will refer to these as a single cross because it is between the same parental individuals.

For this set of crosses we were interested in the effect that population of origin for maternal and paternal plants had on the progeny sex ratios. We used the female frequency of each cross as the continuous response variable. We used the paternal and maternal populations of origin as fixed effects and the individual parents as random effects to determine whether female frequency in the progeny arrays changed depending on the populations participating in the cross.

We also examined whether distance between parental populations had an effect on the female frequency of the progeny array. We used distance between parental populations as a

continuous explanatory variable to determine whether there was a relationship between the progeny sex ratio and distance between the parental populations of origin.

Trait measurements among populations

We measured additional quantitative traits in the progeny to determine the contribution of different populations to the variation in the trait of interest. We measured a suite of traits in the seeds generated from the crosses, including germination rate, height, number of flowers, and number of leaves. We tested for significant differences in these traits between progeny arrays by first calculating a mean value for all traits within a cross. We then performed the same analysis as above. We used the mean trait value as the response variable, and paternal and maternal populations of origin as fixed effects and the individual parents as random effects. We also tested the effect of distance between parental populations by using this distance as a continuous explanatory variable and the trait of interest as the response variable. All analyses were performed with the *lme4* package (Pinheiro et al. 2016) in R v.3.3.2 (R Core Development Team, 2017).

Seedling germination and progeny scoring

To measure traits and sex progeny from the crosses, we planted all seeds produced in the crosses. Seeds were scarified, soaked in water, and placed in a 4°C cold room for 2 weeks. We then planted all seeds in 2” seedling plugs filled with Fafard soil. Planted seeds were left on a misting bench in a greenhouse and misted every hour. We recorded germination data and vegetative traits. *G. maculatum* plants generally do not flower in their first season, so after 1 month of growth we cut back above ground biomass and placed seedlings in a dark cold room

for 6 weeks. Plants were then removed from the cold room, transplanted to 4" pots and kept in the greenhouse. We then measured height, number of leaves, and number of flowers. We also recorded the sex of flowering seedlings. Hermaphrodites can be identified visually by their anthers that produce viable pollen, whereas females produce only aborted filaments and anthers that do not contain pollen.

Results

Within population crosses

There were a total of 402 seeds that germinated and flowered from the within population crosses. The mean germination rate was 0.47 (SE 0.16); there were no significant differences in the germination rate between crosses ($F_{58,400}$=0.54, p=0.49). The average female frequency found in the arrays was 0.29 (SE 0.006). There were no significant differences in the female frequency of the progeny arrays between ($F_{58,400}$=0.77, p=0.76). A histogram of the female frequencies in the progeny arrays shows that there was little variation in the sex ratio between crosses (Fig. 5.1).

Among population crosses

The average female frequency in the progeny arrays was 0.22 (SE 0.006). There was no effect of the sex ratio of the paternal population (R^2=-0.060, p=0.16) or maternal population (R^2=-0.05, p=0.87). There was no relationship between the distance between populations of origin of the parental plants and the progeny array female frequency (R^2=-0.06, p=0.92).

Among population trait measurements

There were no significant differences between parental populations of origin and the progeny array sex ratio in number of leaves, number of flowers, or height (leaves, $F_{8,11}$=0.89, p=0.54; flowers, $F_{8,11}$=0.92, p=0.62; height, $F_{8,11}$=1.20, p=0.36). The average height for seedlings at 3 weeks was 6.4 cm (SE 0.07 cm). The average number of leaves was 5 (SE 1.2), and the average number of flowers was 3 (SE 0.80). There was a pattern with regards to germination rate, in which germination rate decreased slightly when the parental populations of origin were more geographically distant (Fig. 5; R^2=0.53, p<0.01). However, the germination rate was unaffected by the paternal or maternal population of origin (maternal, $F_{4,11}$=0.80, p=.55; paternal, $F_{4,11}$=1.38, p=0.30).

Discussion

In the within population crosses, we did not see a significant effect of either parent. This is somewhat surprising, as many other studies have found significant effects of one or both parents. For example, a study of the genetic control of male sterility in *Thymus vulgaris* found significant effects of the maternal parent and the maternal*paternal parent interaction, which supported a cyto-nuclear model of sex determination (Belhassen 1991). However, the reason for our results could be the sex of the maternal parent that we used. In all crosses performed, we used a female individual as the maternal parent. The female has the male sterility element, and potentially it is the same locus for all individuals. When then crossed with a hermaphrodite, the female frequency will be about equal across all arrays because the male sterility locus in the maternal parent does not vary. Therefore, we were not able to detect the contribution of the maternal parent. Had we used hermaphrodite maternal parents, we may have seen more variation

in the progeny sex ratios, for the following reasons: Assuming cyto-nuclear control, a hermaphrodite phenotype can be achieved in two ways, either the individual has a male sterility locus and a corresponding restorer, or the ancestral genotype of no male sterility element (Frank 1989). If there is variation in the genotype between parents, the progeny sex ratios will reflect this, potentially generating more informative results. However, there is a trade-off, which is that we do not know the genotypes of the hermaphrodites, and it is possible that neither hermaphrodite parent would have male sterility elements, and all progeny arrays would be entirely hermaphrodite. In the among population crosses, we did not find a difference in the progeny sex ratios depending on the paternal population of origin. Most likely this was due to small sample sizes, and so we did not have sufficient power to detect significant differences.

We did not find an effect of distance; there was no relationship between the progeny sex ratio and the distance between the parental populations. This is different than what Van Etten (2009) found. In that study, the frequency of an intermediate phenotype (the individual switched from one sex in the first season to the other sex in the next season) increased with increasing distance between parent populations, and the frequency of hermaphrodites decreased. We did not identify any intermediates in this study. It is possible that the seedlings need to flower another season in order to identify any individuals that switch sex. Additionally, the crosses in Van Etten (2009) that generated intermediates were between hermaphrodites. The among population crosses were completed between a hermaphrodite and a female, and therefore may have the same problem as the within population crosses in that the male sterility elements may be fixed in the female parents; we may only be able to see an interaction between different male sterility elements when crosses are completed between hermaphrodites.

Table 5.1. Populations used in the crosses in this study.

Population	*% female*	*State*	*GPS coordinates*
SU	32	GA	34.77438, -84.67049
SR2	34	NC	35.422394, -82.922287
BCSP	28	VA	37.532272, -78.274544
TSF	18	NY	41.561447, -77.390251
R23	8	NY	42.200165, -73.926727

Table 5.2. Crossing design for the within population crosses. H or F indicates the sex of the plant, hermaphrodite or female; A represents the population of origin; and 1-4 indicate the individual used in the cross. Individuals used as the pollen parents in the cross are along the first row and ovule parents are in the first column. This design was repeated for all 5 populations.

	A_H_1	A_H_2	A_H_3	A_H_4
A_F_1				
A_F_2				
A_F_3				

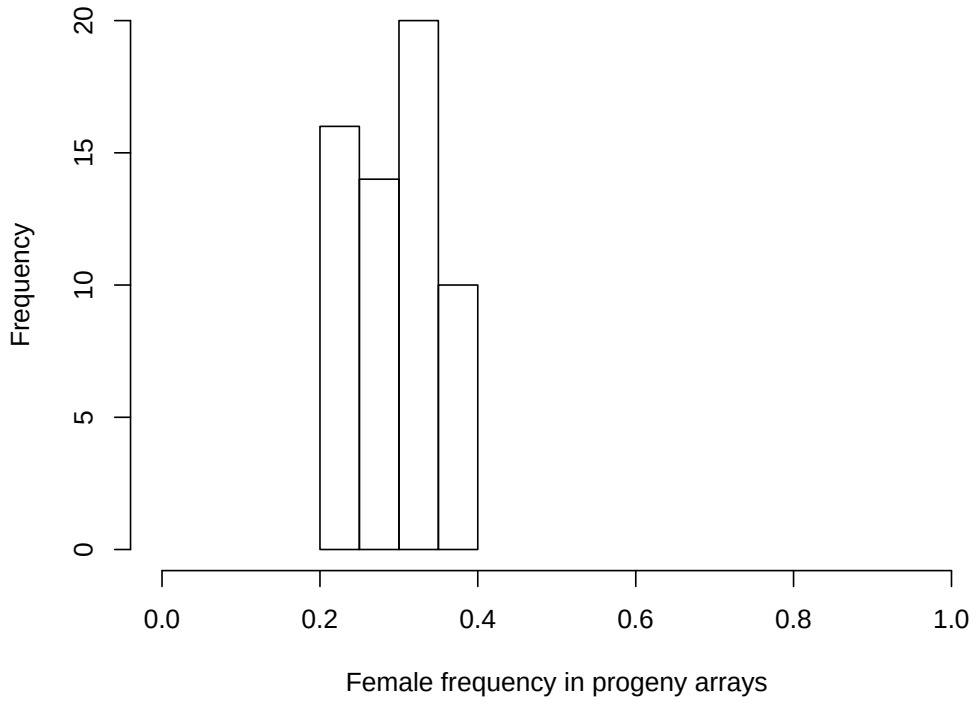

Fig. 5.1. Distribution of sex ratios (female frequency) for the within population crosses.

References

Agren, J., and M. F. Willson. 1991. Gender Variation and Sexual Differences in Reproductive Characters and Seed Production in Gynodioecious Geranium maculatum.

Ashman, T.-L., and C. J. Majetic. 2006. Genetic constraints on floral evolution: a review and evaluation of patterns. Heredity (Edinb). 96:343–52.

Barrett, S. C. H. 2002. The evolution of plant sexual diversity. Nat. Rev. Genet. 3:274–84.

Barrett, S. C. H., and P. R. S. B. 2013. The evolution of plant reproductive systems: how often are transitions irreversible?

Belhassen. 1991. Complex determination of male sterility in Thymus vulgaris L.: genetic and molecular analysis. Theor. Appl. Genet. 82:137–143.

Chang, S. 2007. Gender-Specific Inbreeding Depression in a Gynodioecious Plant , Geranium maculatum. Am. J. Bot. 94:1193–1204.

Dudle, D. a., P. Mutikainen, and L. F. Delph. 2001. Genetics of sex determination in the gynodioecious species Lobelia siphilitica: Evidence from two populations. Heredity (Edinb). 86:265–276.

Etten, M. L. Van, A. C. Deen, J. L. Hamrick, and S. Chang. 2014. Mating system contributes only slightly to female maintenance in gynodioecious Geranium maculatum (Geraniaceae). 113:464–470.

Frank, S.A. 1989. The evolutionary dynamics of cytoplasmic male-sterility. American Naturalist 133:345-376.

Richards, A. J. 1997. Plant Breeding Systems. 2nd ed. Garland Science.

Stamp, N.E. and J.R. Lucas. 1983. Ecological correlates of explosive seed dispersal. Oecologia 59:272-278.

Sun, M. and F.R. Ganders. 1986. Female frequencies in gynodioecious populations correlated with selfing rates in hermaphrodites. AJB 73: 1645-1648.

Taylor, D. R., M. S. Olson, and D. E. Mccauley. 2001. A Quantitative Genetic Analysis of Nuclear-Cytoplasmic Male Sterility in Structured Populations of Silene vulgaris.

Weller, S. G., a K. Sakai, T. M. Culley, L. Duong, and R. E. Danielson. 2014. Segregation of male-sterility alleles across a species boundary. J. Evol. Biol. 27:429–36.

CHAPTER SIX:

CONCLUSION

Many sexual systems have evolved across the angiosperm phylogeny; after hermaphroditism, gynodioecy is the most common sexual system, with 7% of plant species exhibiting this system (Renner and Ricklefs 1995). Through theoretical and empirical work beginning with Darwin (1877), research has shown gynodioecy to be a transitional stage in the evolution of dioecy from hermaphroditism (Barrett 2013). In the first stage of this transition, a male sterility mutation invades a population, resulting in female individuals in the population. Females, to be maintained, must make up the fitness deficit caused by a lack of pollen production; they can persist in populations if they have higher seed fitness than hermaphrodites. This higher fitness can come in the form of more seeds or better quality seeds. Hermaphrodites of gynodioecious species are often self-compatible (in fact, David Lloyd argued that self-compatibility is a necessity for females to remain in populations (Lloyd 1975)). Because of this, inbreeding depression in hermaphrodites can be quite high (Mutikainen and Delph 1998); females, because they necessarily outcross, experience reduced inbreeding depression and so can make up the fitness deficit in producing better quality progeny.

Once females establish, their presence is predicted to exert selective pressure on hermaphrodites. Specifically, the increased ovules available from females puts pressure on hermaphrodites to increase allocation to male traits such as pollen production. If this comes with a concurrent decrease in allocation to female reproductive structures, hermaphrodites will become functionally more male, which allows a female sterility mutation to invade successfully,

leading to a subdioecious population (Bawa 1980; Rosas and Domínguez 2009; Spigler and Ashman 2012).

A unique aspect of gynodioecious species is the wide variation in population sex ratio. In most species with sexual systems other than hermaphroditism, the different mating morphs are held at equal frequency due to negative frequency dependent selection (Barrett et al. 2010). In gynodioecious species, however, female frequency can range from 0 to almost 0.90. This unique aspect of gynodioecious systems allows us to ask questions about how large-scale, among population factors and local, within population forces interact with and change based on the population sex ratio variation we observe.

In Chapter Two, I examined patterns of gene flow between populations over a large geographic region. Non-selective forces such as gene flow have been shown to affect the distribution of a sexual polymorphism (Hodgins and Barrett 2007; Zhou et al. 2012; Yakimowski and Barrett 2014). We might therefore predict that these non-selective factors might interact with the distribution of females across populations of *G. maculatum*. I found that populations with high female frequency (>0.30) had decreased gene flow and migration rates relative to hermaphrodite-only or low female frequency populations.

In Chapter Four, I estimated the distance that pollen is dispersed within populations that vary in their female frequency. I found a similar pattern within populations as I found on a large, among population scale: high female frequency populations had restricted gene flow within the population: pollen dispersal distances were significantly less in populations with high female frequency. High female frequency populations also showed spatial structuring of the sexes, in which the sexes were clustered within the population. This is a common pattern in gynodioecious populations (see Mccauley and Taylor 1997; Dufay and Pannell 2010; Sanderson et al. 2016).

These two factors, restricted gene flow and spatial structure of the sexes, can have implications for selective pressures on females and hermaphrodites and reproductive trait variation between the two sexes. In Chapter Three, I measured traits important for hermaphrodite and female fitness in ten natural populations for three years. I found that hermaphrodites do not increase allocation to male function in high female frequency populations. This may be because, due to the short pollen dispersal distances, hermaphrodites do not experience the population level sex ratio, but rather the local neighborhood sex ratio, and since the sexes are spatially structured, a hermaphrodite's neighborhood female frequency is lower than the population female frequency. In these natural populations, there were not high levels of female compensation. Theoretical predictions state that, under nuclear control of male sterility, females should produce twice the number of seeds of hermaphrodites in order to be maintained (Charlesworth and Charlesworth 1978). Female seed production was this high. However, rhizome length was significantly greater for females in high female frequency populations, suggesting that perhaps they have higher lifetime fitness; perhaps they live longer or are able to flower and set seed even under harsh conditions.

In Chapter Five, I used a quantitative genetic approach to examine the contribution of maternal and paternal factors to the female frequency of progeny arrays generated from crosses completed between females and hermaphrodites within and among populations. There was a significant effect of the paternal population of origin as well as paternal population female frequency: female frequency in the progeny arrays was significantly higher. The results from this research suggest that the dynamics of gynodioecy in *G. maculatum* are complex and involve the interactions between many factors. In particular, the similar patterns of gene flow among

populations and pollen flow within populations suggests that the presence of females increases genetic structure at different spatial scales.

References

Barrett, S. C. H., and P. R. S. B. 2013. The evolution of plant reproductive systems: how often are transitions irreversible?

Barrett, S. C. H., S. B. Yakimowski, D. L. Field, and M. Pickup. 2010. Ecological genetics of sex ratios in plant populations. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 365:2549–57.

Bawa. 1980. Evolution of dioecy in flowering plants. 11:15–39.

Charlesworth, D., and B. Charlesworth. 1978. A model for the evolution of dioecy and gynodioecy. 112:975–997.

Dufay, M., and J. R. Pannell. 2010. The effect of pollen versus seed flow on the maintenance of nuclear-cytoplasmic gynodioecy. Evolution (N. Y). 64:772–784.

Hodgins, K. a, and S. C. H. Barrett. 2007. Population structure and genetic diversity in tristylous Narcissus triandrus: insights from microsatellite and chloroplast DNA variation. Mol. Ecol. 16:2317–32.

Lloyd, D. G. 1975. The maintenance of gynodioecy and androdioecy in angiosperms. Genetica 45:325–339.

Mccauley, D. E., and D. R. Taylor. 1997. Local population structure and sex ratio: evolution in gynodioecious plants. Am. Nat. 150.

Mutikainen, P., and L. F. Delph. 1998. Inbreeding depression in Lobelia siphilitica: among-family differences override between-morph differences. Evolution (N. Y). 52:1572–1582.

Renner, S. S., and R. E. Ricklefs. 1995. Dioecy and Its Correlates in the Flowering Plants. Am. J. Bot. 82:596.

Rosas, F., and C. a Domínguez. 2009. Male sterility, fitness gain curves and the evolution of gender specialization from distyly in Erythroxylum havanense. J. Evol. Biol. 22:50–9.

Sanderson, B. J., M. E. Augat, D. R. Taylor, and E. D. B. Iii. 2016. Scale dependence of sex ratio in wild plant populations : implications for social selection. 1411–1419.

Spigler, R. B., and T.-L. Ashman. 2012. Gynodioecy to dioecy: are we there yet? Ann. Bot. 109:531–43.

Yakimowski, S. B., and S. C. H. Barrett. 2014. Variation and evolution of sex ratios at the northern range limit of a sexually polymorphic plant. J. Evol. Biol. 27:1454–66.

Zhou, W., S. C. H. Barrett, H. Wang, and D. Z. Li. 2012. Loss of floral polymorphism in heterostylous luculia pinceana (rubiaceae): A molecular phylogeographic perspective. Mol. Ecol. 21:4631–4645.