ABSTRACT

Title of Dissertation: NATURAL SELECTION, POPULATION GENETICS, AND TRAIT DIVERSIFICATION OF *SILENE STELLATA* AND ITS POLLINATING SEED PREDATOR *HADENA ECTYPA*

Juannan Zhou, Ph.D., 2017

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My dissertation explores four aspects of the interaction system consisting of the hermaphroditic plant *Silene stellata* and its pollinating seed predator *Hadena ectypa* in a community context. My overarching goal is to deepen our understanding of the selection dynamics influencing floral evolution of hermaphroditic plants.

First, I characterized the mating system of *S. stellata* to evaluate its role on floral evolution of *S. stellata* and the *Silene-Hadena* interaction. Second, I compared the spatial genetic structures of *S. stellata* and *H. ectypa* to evaluate any discrepancy in their dispersal abilities. Third, I addressed whether selection pressures on floral traits of *S. stellata* differ between sexual functions and between pollinator types. Last,

I quantified the genetic basis of the *Silene* floral traits to predict evolutionary response under complex selection scenarios.

In Chapter 1, I found the study *S. stellata* population to be predominantly outcrossing with short pollen dispersal distance. The lack of effect of pollinator types (specialized seed predator and other nocturnal copollinating moths) on *S. stellata* mating system parameters suggests that the dual pollinator type relationship with *S. stellata* is stable and perhaps contributes to the persistence of the plant species. In Chapter 2, I found no genetic differentiation among the *Hadena* populations, while the *Silene* populations showed strong spatial structure. This suggests that pollen flow between *Silene* populations rarely co-occurs with moth movement. This asynchrony in gene flow could potentially stabilize the interaction dynamics and prevent strict local coadaptation. In Chapter 3, I found conflicting selection pressures between male and female reproductive functions of *S. stellata*. Strong selection through female function was detected to avoid fruit predation, while competition for mates through male function provides a counterbalancing force potentially contributing to the long-term maintenance of this interaction. In Chapter 4, I found intermediate heritability and prevalent positive genetic correlations between *Silene* floral traits, suggesting the *Silene* population is capable of responding to phenotypic selection on its floral design, while the abundant genetic correlations could also pose certain constraints on trait divergence.

My results suggest that floral evolution is governed by complex, interdependent processes and that the *Silene-Hadena* interaction could be maintained through the dynamical balance between various opposing evolutionary forces.

NATURAL SELECTION, POPULATION GENETICS, AND TRAIT DIVERSIFICATION OF *SILENE STELLATA* AND ITS POLLINATING SEED PREDATOR *HADENA ECTYPA*

by

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Dedication

This dissertation is dedicated to my family.

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Chapter 1: Characterization of the mating system of the perennial tetraploid herb *Silene stellata.*

Abstract

Plant mating systems determine the transmission of genes across generations. Selfing and correlated mating increase the relatedness between siblings and reduce the effective size of plant populations, and therefore are important population parameters. In this paper, we characterized the mating system of a perennial herb *Silene stellata*. We used highly variable microsatellite markers to explicitly resolve paternity of the sampled progeny. We found the study population of *S. stellata* to be predominantly outcrossing (outcrossing rate, $t = 0.83$) with a correspondingly high magnitude of inbreeding depression through selfing (50% fitness decline). We also detected significant correlations in both selfing and outcrossed paternity at the fruit and maternal family level and found the average pollen dispersal distance to be limited (mean=3.9 m).

Our results suggest that although herkogamy and dichogamy of *S. stellata* can potentially reduce self-fertilization within flowers, geitonogamous pollen transfer appears to be a common mechanism of within plant selfing. We found one trait, anther-stigma separation with significant positive effect on outcrossing rate, suggesting the importance of herkogamy in preventing within-flower selfing. We found no significant effects of year and pollinator types on outcrossing rate. Correlated paternity suggests that seeds from the same fruit and/or plants are sired by a limited number of pollen donors, which

could be the direct result of low pollen dispersal and male-male competition, with the latter magnified by the limited ovule number per pistil.

Introduction

Plant mating systems define the mode of gene transmission, and thus have important consequences for many evolutionary phenomena (Charlesworth et al. 1990; Barrett and Charlesworth 1991; Byers and Waller 1999). In partially outcrossing plant species, self-fertilization produces higher genetic relatedness between siblings than expected under random outcrossing (Ritland 1989). Increased selfing may compromise female fitness for hermaphroditic plants through inbreeding depression in the progeny (Charlesworth and Charlesworth 1987). As a consequence, self-compatible hermaphrodites often show complex mechanisms to promote outcrossing (Darwin 1877; Barrett 2002). The intrafloral mechanism of dichogamy (temporal separation in pollen dehiscence of stamens and stigmatic receptivity of pistils) and herkogamy (spatial separation between anther dehiscence and stigma receptivity) are thought to be effective in reducing interference between male and female function and avoiding self-fertilization within hermaphroditic plant species (Lloyd and Yates 1982; Lloyd and Webb 1986; Webb and Lloyd 1986). However, asynchrony among flowers in the inflorescence can create the opportunity for selfing between flowers on the same plant (geitonogamy) (Dudash 1991; Barrett 2002).

Traditional mixed mating models assume that outcrossed individuals in each fruit or maternal family/plant are derived from independent pollination events and that all plants have equal outcrossing rates (Clegg 1980; Brown et al. 1985). However, ecological, demographic, and genetic factors often lead to deviations in the mating system from this assumption (Morgan and Barrett 1990). Outcrossing rates can vary

among individual plants and fruits within a plant via both self and outcross mating events (Ritland 1989; Morgan and Barrett 1990) as a result of ecological factors such as local plant density (Ellstrand et al. 1978; Ellstrand and Foster 1983; Ward et al. 2005; Naito et al. 2008), floral phenology (Kameyama and Kudo 2009), pollinator types (Brunet and Sweet 2006), and floral trait variation among individuals (Schoen 1982; Marshall and Abbott 1984; Glover and Barrett 1986; Takebayashi et al. 2006; Williams 2007).

Furthermore, various other mechanisms including limited pollen dispersal or pollen carryover (Thomson and Plowright 1980; Schmitt 1983; Fenster 1991*a*), and male-male competition (Morgan and Barrett 1990) may also contribute to the correlation of paternity within progeny arrays. The level of correlated paternity corresponds to the probability that two siblings within a fruit or maternal family have the same pollen donor (Ritland 1989). Correlated paternity can be regarded as the inverse of an effective pollination neighborhood size, a measure analogous to Wright's neighborhood size (Austerlitz and Smouse 2001). The level of outcrossing, correlated selfing, and correlated paternity increase inbreeding and kinship within families, leading to smaller effective population size than expected under random mating (Ritland 1989), and thus significantly contributing to genetic drift. Furthermore, correlated mating may also reduce the effectiveness of selection among siblings (Karron and Marshall 1990).

Despite the importance of plant mating system in diverse evolutionary phenomenon, there are relatively few studies that have characterized mating system to the level of paternity. Species of the genus *Silene* exhibit a high diversity of sexual systems with hermaphroditism being the most common followed by dioecy, gynodioecy, and gynodioecy-gynomonoecy (Casimiro-Soriguer et al. 2015) and have a long history

serving as model systems for the study of sexual and mating system evolution (Kephart et al. 2006; Bernasconi et al. 2009).

In this study, we characterized the mating system of the North American tetraploid, hermaphroditic long-lived perennial *Silene stellata* (Caryophyllaceae). *Silene stellata* exhibits a nocturnal pollination syndrome with white, protandrous flowers that are pollinated by two major groups: the obligate seed predator, *Hadena ectypa,* and a number of equally effective purely mutualistic generalist moth species (Reynolds et al. 2009). Using explicit paternity assignment test, we asked the following questions: (1) What is the outcrossing rate of *S. stellata*? Does outcrossing rate differ between years and pollinator types? Does the estimated outcrossing rate correspond to expected levels of inbreeding depression; (2) Does selfing and outcrossing occur non-independently within fruits and maternal families; (3) What is the distribution of pollen dispersal distance and its contribution to the observed pattern of correlated mating? (4) How does individual variation in floral design variation influence individual outcrossing rates?

Methods

Study species

Silene stellata L. is an iteroparous, long-lived perennial herb that is distributed throughout the eastern United States. Flowering occurs from early July through early September. It produces panicle inflorescences with white, hermaphroditic, protandrous flowers (average of 25 ovules/pistil, Reynolds et al. 2009). A plant usually produces multiple stems and on average ~40 flowers each flowering season (Reynolds et al. 2012).

A previous study using insect exclosure cages documented modest seed-set in the absence of pollinators and provided an outcrossing rate estimate of 73% (Reynolds 2008; Reynolds et al. 2009)

Silene stellata is pollinated by its obligate pollinating seed predator moth *Hadena ectypa* as well as by a number of generalist moths including *Amphipoeaea americana*, *Feltia herelis*, *Autographa precationis*, *Cucullia asteroids*, *Halysidota tessellaris*, *Lochmaeus manteo* (Reynolds et al 2009). Adult male and female *H. ectypa* extract nectar in the flowers of *S. stellata* with pollination taking place simultaneously. Oviposition behavior follows nectaring, as female moths lay single or multiple eggs at the base of the ovary or on the ovary wall (Zhou *et al.* 2016). A larva can consume up to 40 flowers and/or unhardened fruits under lab conditions (Reynolds et al. 2012). *Hadena ectypa* and the copollinators are equally effective at pollen transport (Reynolds et al. 2009) and exhibit consistent temporal separation and serve as the primary pollinators for *S. stellata* in the early and late flowering season, respectively (Zhou *et al.* 2016).

Field experiments

In 2012 and 2013, we conducted field experiments in an open meadow under a power line cut near the University of Virginia's Mountain Lake Biological Station in Giles County, Virginia, U.S.A. (37.3471, -80.5426, elevation ≈ 1,100–1,300 meters).

We constructed an experimental plot (20m × 20m) that was enclosed with 6-foot fencing to prevent deer herbivory, near the top of a naturally occurring population. In each year, two experiments were conducted during the *Hadena*-dominant and the copollinator-dominant periods (referred to as "early" and "late" hereafter). Each experiment was carried out for a week with an experimental population consisting of ~60

equally spaced adult plants (at least 1 m separated all flowering plants in each time period). Within the exclosure, all flowering non-experimental plants had their above ground stems removed. Additionally, in order to prevent pollen flow from outside of the study population, a 10-meter exclusion zone around the exclosure was created by removing all flowering stems of *S. stellata* plants in this area. From earlier work we knew that *S. stellata* pollen carryover distances are 1-2 meters based on a fluorescent dye survey (Reynolds et al. 2009). Thus the exclusion zone was expected to prevent the majority of pollination by non-sampled plants from outside the exclosure, so that the majority of potential pollen donors were restricted to the experimental plants. We divided the plot into 8 transects along the elevation gradient, with each transect consisting of 8 evenly spaced plants. To avoid repeated measurements, plants that were used in previous experiments or in the previous year were excluded from later experiments. Due to seasonal plant mortality, on average ~57 plants were used per experiment (2012 early N = 59; 2012 late N = 58; 2013 early N = 55; 2014 late N = 55). All flowers opened during the one-week experimental period were tagged. We harvested all labeled reproductive units (RUs) from the study plants after fruit maturation, but before fruit dehiscence.

Paternity assignment

We used paternity assignment tests to directly estimate mating system parameters using 11 highly variable microsatellite markers (Zhou *et al.* 2016). Leaf tissue was collected and extracted for genomic DNA for a total of 227 adult *S. stellata* plants. To obtain sufficient amounts of progeny genomic DNA for subsequent amplifications, the seeds from all successful fruits were first sowed and cold stratified during the winter of 2012 and 2013, then germinated in a greenhouse environment in the following springs,

respectively. Seedlings of 2012 were harvested into silica gel for subsequent DNA extraction, while DNA of seedlings of 2013 were preserved on the Whatman FTA® cards (Whatman Inc., USA), following the manufacturer's instruction. Due to seed predation by *H. ectypa* larvae, only 42 percent of the tagged flowers yielded mature fruits without herbivory and with at least one seed. A total of 2371 seedlings were successfully germinated and preserved (average number of seedlings collected per plant = 10.44).

Adult plant and the 2012 seedling DNAs were extracted using the Autogen Plant Kit, following the manufacturer's protocol. DNAs were extracted from the 2013 seedlings using the FTA® purification reagent (Whatman Inc., USA) under a modified extraction protocol (Siegel et al. 2017). Plants were genotyped with 11 novel microsatellite loci designed for *S. stellata* (Zhou *et al.* 2016). DNA amplification was performed with fluorescently labeled forward primers (FAM- or HEX-) following the protocol described in Zhou *et al.* (2016). We carried out fragment analysis of PCR products on an ABI 3730xl automated capillary sequencer at the Laboratories of Analytical Biology (LAB) of the Smithsonian National Museum of Natural History. Fragment patterns were visualized and scored using the GeneMapper V3.7 software (Applied Biosystems, Foster City, CA, USA). Continuous product sizes were exported and rounded to multiples of repeat number (binned) using the software Tandem (Matschiner and Salzburger 2009).

We assigned paternity of individual seedlings categorically to the candidate sires using the paternity exclusion program PolyPatEx (Zwart et al. 2016). The set of candidate sires consisted of all study plants of a given experimental period. Given that *S. stellata* is a tetraploid, the underlying genotypes of partial heterozygotes cannot be readily resolved

(for example, an individual showing two alleles A, B on a given locus can have AAAB, AABB or ABBB as the underlying genotype). PolyPatEx is designed for polyploids and resolves this challenge by assessing the genotypic compatibility between the progeny, the mother, and the candidate sires through the enumeration of all possible allelic configurations given their allelic phenotypes (presence or absence of alleles). Paternity analysis using "PolyPatEx" was carried out in the R environment (R Core Team 2016). We used the number of seedlings sired as the measure of male fitness in subsequent statistical analyses. Female reproductive success of individual plants was measured as the number of successful fruits (fruits that were not subjected to *H. ectypa* predation and matured at least one seed; see Chapter 3).

Mating system analysis

Individual outcrossing rates were calculated as one minus the ratio of progeny resulting from selfing to the total number of progeny genotyped for each plant. To obtain estimates of population level outcrossing rate and confidence interval, we bootstrapped outcrossing rates of individual plants with 9999 iterations. Population level outcrossing rate was estimated for the four experiments separately. Bootstrapping was performed in the R environment (R Core Team 2016) using the package "boot".

We further characterized the *S. stellata* mating system using Ritland's correlated mating framework (Ritland 1989) which assumes two individuals sampled from the same progeny array (within the same fruit or maternal family) may result from non-independent mating events. Using resolved paternity, we estimated two parameters analogous to the Ritland's selfing correlation (r_S) and paternity correlation(r_P) (Ritland 1989): r_S measures the correlation of selfing between two siblings within a progeny array,

while r_P measures the probability that two outcrossed individuals randomly chosen within a progeny array are sired by the same individual. We estimated the correlated mating parameters on two hierarchical levels by sampling sibling pairs from within and between fruits. We estimated r_S by bootstrapping pairs of siblings on the two hierarchical levels using 9999 iterations. The statistic calculated was the covariance of selfing divided by s[1-s] (Ritland 1989), where s is the selfing rate in the sample. We estimated r_P by bootstrapping pairs of outcrossed sibs within and between fruits with the statistic being the proportion of full-sib pairs among all outcrossed sibling pairs (Ritland 1989).

To compare female and male reproductive success (RS), we quantified the correlation between female and male fitness using a Spearman rank sum test. Individual fitness scores were transformed to proportions of population fitness equaling the ratio of individual female (number of successful fruits) or male fitness (number of seeds sired through outcrossing) to the total female (total number of successful fruits) or male fitness (total number of seedlings genotyped) of each experiment. We divided individual male and female fitness by the population sum to put the two fitness components on the same scale. The correlation coefficients were identical to those calculated based on absolute fitness scores (data not shown).

Assessing inbreeding depression

We used the inbreeding coefficient (F) together with the selfing rate (s) to estimate inbreeding depression (ID) from seed to reproductive maturity in our study population. Estimation of the inbreeding coefficient, F, of the 227 study plants was carried out in Genodive (Meirmans and Van Tienderen 2004*a*). We assessed the significance of the F

estimate by performing 999 bootstraps. The bootstrap distribution of F was used in subsequent estimation of inbreeding depression values.

At equilibrium, the expected value of F = sw/(2-2s+sw) (Goodwillie et al. 2005), where w is the fitness of the selfed individual relative to an outcrossed individual, and s is the selfing rate. ID = 1 – w, which measures the deduction in fitness from seed to reproductive maturity of selfed individuals proportional to the lifetime fitness of outcrossed individuals. Based on the above identities, we used Ritland's equilibrium estimator: ID = 1 - 2[(1-s)F/s(1-F] (Ritland 1990). We generated 95% bootstrap percentile confidence intervals for the estimation of ID by resampling (s, F) with 999 iterations from the empirical distribution of s and the bootstrap distribution of F generated using Genodive.

Regression analysis

We used a generalized linear model (GLM) to test for (1) difference in outcrossing rates between pollinator types and across years; and (2) potential contribution of floral design to individual outcrossing rates. The GLM model included the main effects of year, pollinator type and their interaction as well as eight plant trait measures: 1) corolla tube length (TL); 2) corolla tube width (TW); 3) length of the largest petal (PL); 4) width of the largest petal (PW); 5) number of fringes on the distal margin of a randomly chosen petal (FR); 6) anther-stigma separation (AN-ST); 7) number of flowers (NF); and 8) height of the longest stem (HT). All floral traits except anther-stigma separation were measured on flowers during the first day of opening (the male phase). Anther-stigma separation (AN-ST) was measured on the first day of the female phase and was calculated as the difference between nectar-anther distance (distance from the nectary at the base of

the flower to the tip of the anther) and nectar-stigma distance. On average we measured five flowers on each plant. Means of floral-trait measurements from all measured flowers on a plant were calculated to obtain one representative measure for each trait per plant per year. The mean trait measure was z-transformed within each experiment to mean = 0 and variance = 1. Individual outcrossing rates were arc-sine transformed to ensure normality of the residuals (Sokal and Rohlf 1995).

Results

Outcrossing rates of the four experiments were all significantly different from 0 and ranged between 0.78 and 0.91, with an average of 0.83 across experiments (Table 1). One-way ANOVA showed no significant difference in outcrossing rates among experiments ($F=0.09$, $df=1$, $P>0.05$). Selfing rate was significantly positively correlated with outcrossing male fertility (seedlings sired through outcrossing) for the early experiments in both years (2012 Early: Spearman correlation coefficient $\rho=0.76$, $P<0.001$; 2013 Early $\rho=0.60$, $P<0.001$), while the correlation was positive but marginally significant for the two late experiments (2012 Late: $\rho=0.34$, $P=0.073$; 2013 Late: $\rho=0.43$, $P=0.088$). Significant positive correlation between male and female RS was observed in all four experiments except 2013 Late (Figure 1).

When sibling pairs were sampled within fruit, the estimated correlation of selfing (r_S) were all significantly different from zero and ranged between 0.25 and 0.51 (mean r_S across experiments=0.37). Correlation of selfing (r_S) was higher in 2012 than 2013 (Table 1) and r_S remained significant but was much smaller when seedlings were sampled

between fruits (mean r_S across experiments=0.14). A Wilcoxson rank sum test demonstrated that the difference in r_S between the two hierarchical levels (within and between fruit) was significant (W=16, P<0.05).

Similarly, paternity correlation r_P was significantly higher when sibling pairs were sampled from within fruit than between fruits (W=16, P<0.05): r_P=0.35–0.60 (mean r_P=0.41) within fruit, r_P=0.09–0.32 (mean r_P=0.19) between fruits (Table 2). This indicated that on average 41 percent of the sibling pairs sampled within fruit were full-sibs, compared to 19 percent between fruits (Table 2).

Using Ritland's (1990) inbreeding depression estimator and 999 bootstraps, we found the inbreeding depression (ID) from seed to reproductive maturity to be 0.50 (95% CI: -0.42, 1.0). Thus we detect a 50% decline in overall progeny fitness following selfing compared to progeny produced from matings between unrelated individuals.

The distributions of pollen dispersal distances were similar independent of pollinator type, early and late in the season, and in both years (Figure 2). The mean (SE) of pollen dispersal distance was 3.89 m (2.36) when data were pooled across experiments.

We found that only one trait, anther-stigma separation (AN-ST), was associated with outcrossing rate, with a significant positive effect (P=0.044) under the GLM model. The other floral traits, year, pollinator type, and year × pollinator interaction had no significant effect on outcrossing rate (Table 3).

Discussion

The factors influencing mating system parameters have important implications for long-term population persistence. Systems where both an obligate pollinator and more generalized non-obligate pollinators are present, beg the question of how and why this relationship is maintained. Our investigation of a hermaphroditic system using explicit paternity assignment demonstrates that our study population of *S. stellata* is predominantly outcrossing independent of pollinator types across two years; that only one trait, anther-stigma separation has a significant influence on outcrossing rate; and that significant correlated paternity within a fruit is much stronger than between fruits, as would be expected, independent of whether the specialized pollinator, *H. ectypa* is dominant compared to the purely mutualistic moth copollinators. Thus the lack of effect of pollinator types on *S. stellata* mating system parameters suggests that this dual pollinator relationship with *S. stellata* is stable and perhaps protective for the persistence of the plant species.

The average estimate of outcrossing rate across our four studies, 83%, is similar to a previous estimate using allozyme markers (>73%; Reynolds 2008). Our estimate of outcrossing rate is also close to the humming-bird pollinated, closely related species *S. virginica* using allozyme markers (89%; Dudash and Fenster 2001). Similar to *S. virginica*, strong protandry is present in *S. stellata*, which may effectively prevent self-fertilization within a flower. However, Reynolds *et al.* (2009) showed *S. stellata* produced on average 20% seed set under total pollinator exclusion (roughly one third of that under non-exclusion), providing evidence for the potential occurrence of some

within-flower fertilization under natural conditions. The significant effect of anther-stigma separation on outcrossing rate indicates the importance of herkogamy in preventing within-flower selfing, consistent with previous findings (Lloyd and Webb 1986; Webb and Lloyd 1986; Herlihy and Eckert 2002; Takebayashi et al. 2006). Furthermore, the small genetic variation in stigma and anther exertion (Chapter 4) can be explained by strong historical selection for the avoidance of inbreeding depression.

As a long-lived perennial, *S. stellata* produces many flowers throughout the flowering season across many years. Consequently, individuals often have flowers exhibiting compatible sexual functions on the same night, providing the opportunity for pollination between flowers within the same plant, e.g., geitonogamy. Additionally, *H. ectypa* and the copollinators both visit multiple flowers on the same plant (J. Zhou personal observation). Therefore, geitonogamous pollen transfer is likely a causative agent of selfing observed here. All other features of the *S. stellata* flower (temporal and spatial separation of male and female function) are consistent with a nocturnal pollination syndrome (Fenster et al. 2004) that promotes outcrossing, although pollination specialization does not preclude the evolution of floral traits to promote delayed selfing (Fenster and Marten-Rodriguez 2007).

Since perennial plants likely carry higher genetic load than annuals (Barrett and Eckert 1990), high inbreeding depression is expected to be concomitant with low selfing rate in perennials (Stebbins 1950; Jain 1976). The estimated 50% reduction in fitness of self progeny compared to outcross progeny (Ritland 1990), suggests that the magnitude of inbreeding depression is great enough to counter the transmission bias associated with

the ability to both self and outcross in the absence of pollen discounting (Fisher 1941) and promotes outcrossing in this system.

Our indirect estimation of lifetime inbreeding depression (ID=50%) in *S. stellata* using Ritland's equilibrium estimator is also similar to other closely related North American perennial *Silene* species that exhibit herkogamy and dichogamy. For example, in the hummingbird pollinated *S. virginica*, a high outcrossing rate of 89% was found along with a 40% decline in progeny fitness in the greenhouse environment following inbreeding compared to outcrossed progeny (Dudash and Fenster 2001). In *S. caroliniana*, significant inbreeding depression has also been detected on reproductive success also measured under greenhouse conditions (Konkel, Fenster and Dudash unpubl. data). Alternatively, there are also *Silene* species using selfing as the primary reproductive strategy (e.g., *S. douglasii* var *oraria*, Kephart *et al.* 1999; *S. noctifolia*, Davis and Delph 2005). For the endangered *S. douglasii* var. *oraria*, high inbreeding depression co-occurs with moderate outcrossing rate, probably as the result of selection for reproductive assurance (Kephart et al. 1999). Overall, our results of high inbreeding depression described here and in previous studies are consistent with the polyploid genomes of *Silene* evolving diploidization as we would expect limited expression of recessive deleterious alleles following selfing when the genome is polyploid (Bever and Felber 1992).

Our detection of relatively high correlated selfing when sibling pairs were sampled within fruit, indicates that selfing occurs nonrandomly in our system. This may be in part due to the limited number of ovules per pistil (n = 25; Reynolds et al. 2009) and the large number of pollen grains detected following a single pollinator visit (mean

(SE) for *H. ectypa* = 69 (8) and 78 (8) for copollinators; Reynolds *et al.* 2012). Thus there are three times as many pollen grains deposited from a single visit than there are ovules to fertilize. If geitonogamy is the primary mechanism of selfing in *S. stellata*, the high correlated selfing rate might be one consequence of the floral mechanism to limit pollinator visitation. In the dioecious *S. latifolia*, following pollination and oviposition, emission of scent quickly decreases (Dötterl et al. 2005). Scent is an important attractant to *Hadena* moths (Kephart et al. 2006; Prieto-Benítez et al. 2017), preventing further pollinator visitation. Castillo et al. (2013) has also shown a preference for adult *H. ectypa* to deposit eggs into newly opened flowers versus older flowers. In these scenarios where the first pollinator visiting a female-phase flower carries pollen grains from a male-phase flower on the same plant, the plant can act as a major pollen donor to itself, given the large number of pollen grains deposited in one visit. The effect could be more pronounced given the short pollen dispersal distance (Figure 2). However, the consistent relatively high outcrossing rates shown here suggests that pollinators are moving between plants, and plants near one another appear to be unrelated and/or outcross pollen grains are outcompeting self-pollen grains. Research is warranted on whether differential competitive ability of self and outcross pollen grains occurs to help resolve this issue.

As might have been expected, correlated selfing between fruits was roughly half the magnitude as r_S within fruit, although still significantly different from zero. The significant correlated selfing between fruits reflects variation in individual outcrossing rates. Previous studies suggest that variation in individual outcrossing rate may be caused by characteristics of floral design (Glover and Barrett 1986; Ritland 1989), or flowering phenology (Cruzan et al. 1994; Petit et al. 1997; Dudash and Fenster 2001). Here we

found a significant positive effect of anther-stigma separation (AN-ST) on outcrossing rate. This is consistent with the evidence of self-fertilization within flowers in Reynolds *et al.* (2009), as longer anther-stigma separation is expected to decrease the possibility of contact between the male stamens and the female stigmatic surfaces, leading to lower chance of self-fertilization within flowers. Note that due to the relatively small number of seedlings sampled per plant, statistical errors in estimation of individual outcrossing rates could potentially mask subtle effects of floral trait variation. Therefore, future work with larger maternal family sizes and even sampling among fruits is needed to investigate the mechanism promoting outcrossing in *S. stellata*.

Our inbreeding depression estimate of 50% provides support for the hypothesis of a high selective premium to avoid selfing. Although we found only one floral trait (anther-stigma separation) affecting outcrossing rate, our GLM analysis showed no effect of year and pollinator type. Hence, potential selection for enhanced outcrossing is likely to be exerted through floral morphology but less so through specialization to certain groups of pollinators. Future work that quantifies inbreeding depression of *S. stellata* while utilizing more robust methodology of estimating individual outcrossing rates will provide insight into the question of if selection for outcrossing is a dominant force underlying floral adaptation of *S. stellata*.

Our finding of a significant correlation in outcrossed paternity when siblings are sampled within fruit and between fruits, indicates that seeds of maternal plants could be sired by a limited number of pollen donors. Our results are also consistent with previous findings of prevalent correlated paternity in animal-pollinated plants (Dudash and Ritland 1991). Correlation in paternity can arise during various reproductive stages including

pollination, fertilization and seed maturation (Morgan and Barrett 1990). Significant correlation in paternity indicates that there is higher variance in male reproductive success than expected if mating occurs randomly. Our findings (Chapter 3) that male siring ability is significantly associated with several floral traits suggests that some plants may be more attractive to pollinators than others, and thus are better sires. Therefore, male-male competition for mating success is more likely than female choice to be the cause of the high variance in male reproductive success leading to the high paternity correlation. Furthermore, the increased variance in male mating success due to the effect of floral design on pollination attraction indicates the possibility of interaction-independent sexual selection where variance in mating success does not arise from direct interactions between conspecifics, but rather through the interaction with animal pollinators (Murphy 1998; Delph and Ashman 2006). Additionally, the short pollen dispersal distance documented here (Figure 2) could lead to correlated paternity as a maternal parent is only exposed to a limited number of potential pollen donors in its vicinity. Both male-male completion and short pollen dispersal distance could potentially limit the effect of female choice by reducing the pool of potential mates for the female function of a given plant. The positive correlation between male siring ability and selfing rate suggests that pollen discounting, the cost of selfing in terms of male outcrossing ability (Charlesworth 1980), would not be an obstacle to the initial evolution of increased selfing rates.

The short pollen dispersal distances are somewhat contrary to previous work comparing bee and butterfly pollinator flight distances (Schmidt 1981). However, because *H. ectypa* is obligate on *S. stellata*, female *H. ectypa* may be much more

systematic in their flights versus non-obligate moth or butterfly pollinators. We also note that our pollen dispersal distances are likely biased upward due to our experimental design of thinning plants to a one meter grid structure. For example, pollinator flight distance is typically negatively correlated with plant density (Fenster 1991*a*). Limited pollen dispersal distance suggests that the effect of male-male competition on correlated paternity may be undermined. On the other hand, the short pollen dispersal distance may enhance male-male competition via self versus outcross pollen through pollen gamete competition or female choice acting in the pollen-pistil arena (Fenster and Sork 1988; Fenster 1991*b*).

Finally, the high outcrossing rates detected are also consistent with limited population genetic structure found within populations of *S. stellata* (see Chapter 2). The relatively limited pollen dispersal distances that we detect are also consistent with the significant population genetic differentiation (see Chapter 2) and the lack of pollen transfer to *S. stellata* plants isolated by 30 m from the main study population in an earlier study (Kula et al. 2014). In conclusion, our findings are consistent with pollination specialization contributing to high outcrossing rates in the absence of delayed selfing mechanisms.

Table 1. Outcrossing rates for two years in four field experiments conducted early and late in the flowering season of *Silene stellata* in one field site near Mountain Lake Biological Station, Pembroke, VA. Parameter estimation was based on 9999 bootstrap resampling of outcrossing rates of individual plants. N: number of seed plants.

Experiment	N	Outcrossing rate (95% CI)
2012 Early	20	0.85 (0.77–0.97)
2012 Late	28	0.79 (0.71–0.88)
2013 Early	31	0.78 (0.72–0.85)
2013 Late	17	0.91 (0.85–0.98)

Table 2. Estimates of correlated mating parameters (95% CI) for two years in four field experiments conducted early and late in the flowering season of *Silene stellata* in one field site near Mountain Lake Biological Station, Pembroke, VA in 2012 and 2013. Parameters were estimated based on 9999 bootstrap resampling of sibling pairs within fruit and between fruits. r_S : correlation of selfing; r_P: correlation in outcrossed paternity.

	Within Fruits		Between fruits	
	r_S	r_P	r_S	r_P
2012 Early	0.46 (0.41–0.50)	0.60 (0.58–0.62)	0.19 (0.17–0.21)	0.32 (0.31–0.33)
2012 Late	0.51 (0.47–0.55)	0.35 (0.32–0.37)	0.15 (0.14–0.17)	0.17 (0.16–0.18)
2013 Early	0.29 (0.17–0.40)	0.35 (0.28–0.43)	0.07 (0.03–0.11)	0.09 (0.08–0.11)
2013 Late	0.25 (0.19–0.31)	0.36 (0.34–0.39)	0.13 (0.09–0.17)	0.18 (0.17–0.19)
Mean	0.37	0.41	0.14	0.19

Table 3. Results of generalized linear multiple regression testing effects of pollinator types, year, and plant traits on individual selfing rates of *Silene stellata*. Selfing rate was arc-sine transformed. TL: corolla tube length; TW: corolla tube width; PW: petal width; PL: petal length; FR: number of petal fringes; (AN-ST): anther-stigma separation; HT: display height; NF: number of flowers.

	Estimate	SE	t value	P-value
TL	-0.045	0.048	-0.942	0.349
TW	-0.043	0.053	-0.808	0.422
PW	-0.029	0.069	-0.417	0.678
PL	0.016	0.065	0.252	0.802
FR	0.064	0.047	1.342	0.183
(AN-ST)	0.098	0.048	2.052	**0.044**
HT	0.003	0.050	0.052	0.958
NF	0.008	0.051	0.158	0.875
Year	-0.416	0.345	-1.205	0.232
Pollinator	-3.565	2.783	-1.281	0.204
Year × Pollinator	0.292	0.223	1.308	0.195

Figure 1. Correlation of female and male fitness for two years in four field experiments conducted early and late in the flowering season of *Silene stellata* in one field site near Mountain Lake Biological Station, Pembroke, VA. Fitness scores were scaled by the sum of individual fitness of each experiment. Also shown are the Spearman correlation coefficients (ρ) and associated P-values.

Figure 2. Distribution of pollen dispersal distance based on direct paternity assignment for the four field experiments conducted early and late in the flowering season of *Silene stellata* in one field site near Mountain Lake Biological Station, Pembroke, VA. Mean±SE of pollen dispersal distance based on data pooled across years was 3.89±2.36 m.

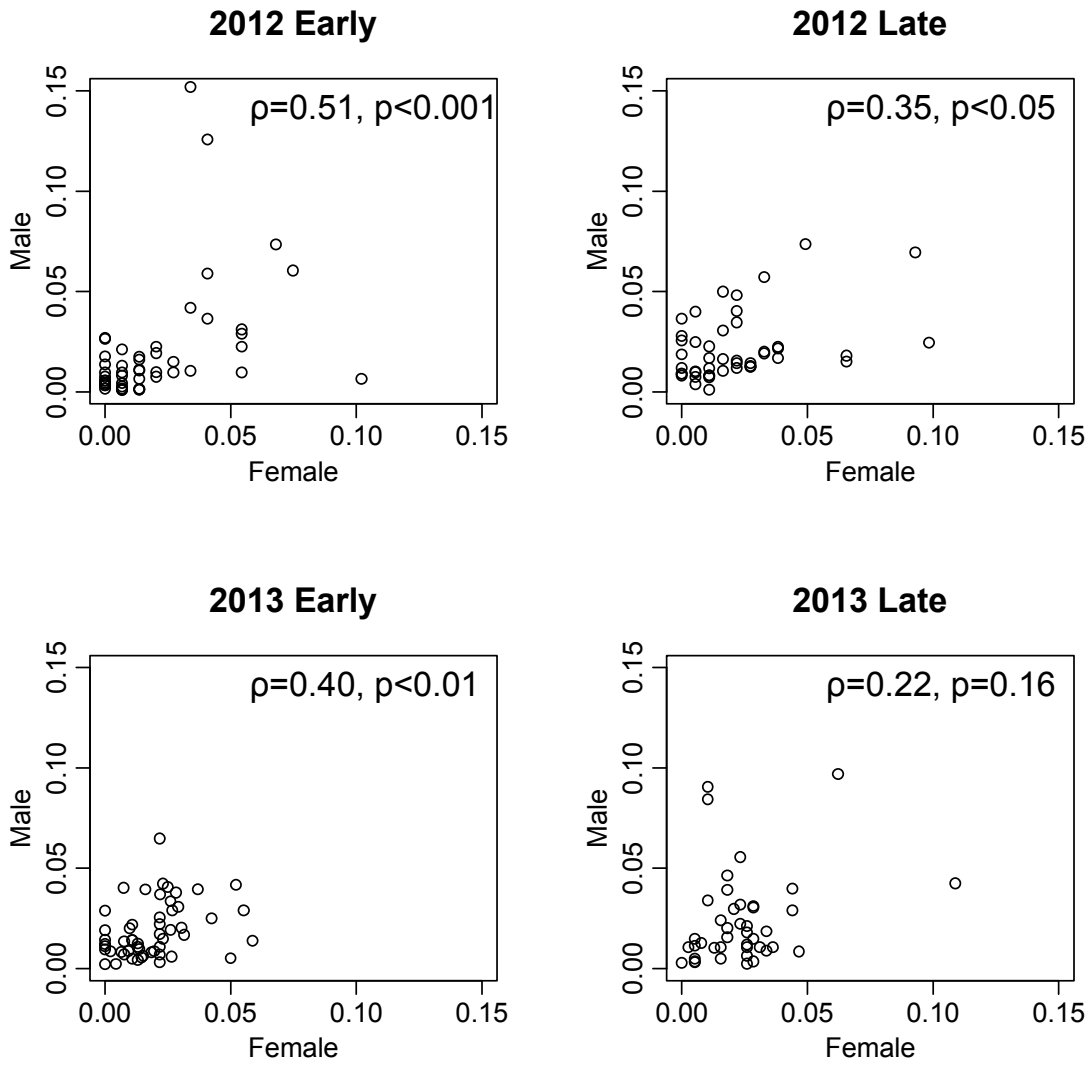

2012 Early
ρ=0.51, p<0.001
2012 Late
ρ=0.35, p<0.05
2013 Early
ρ=0.40, p<0.01
2013 Late
ρ=0.22, p=0.16
Male
Female
0.00
0.05
0.10
0.15

FIG. 1

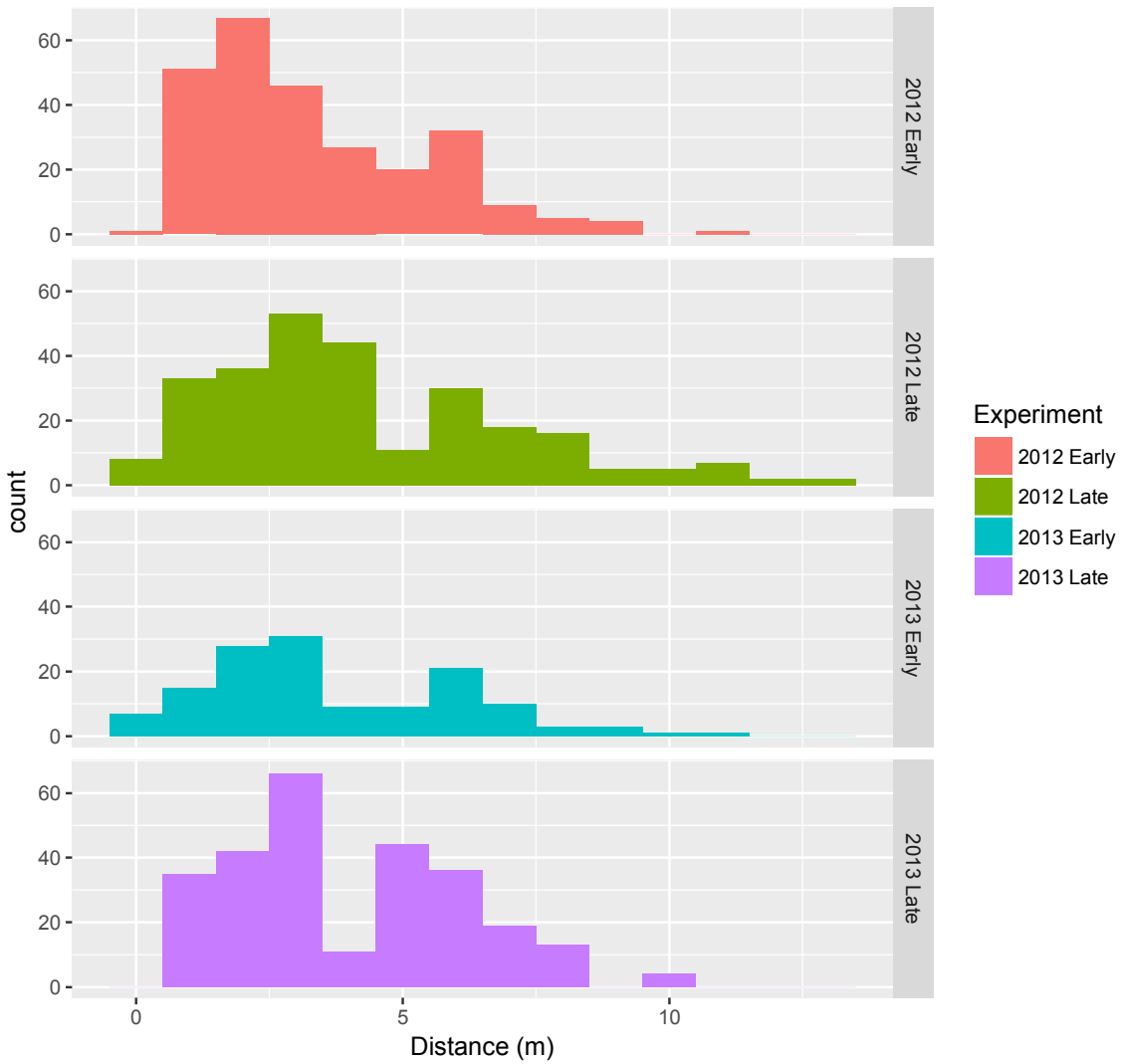

count
60
40
20
0
2012 Early
2012 Late
2013 Early
2013 Late
0
5
10
Distance (m)
Experiment
2012 Early
2012 Late
2013 Early
2013 Late

FIG. 2

Chapter 2: Comparison of population genetic structures of the plant *Silene stellata* and its pollinating seed predator moth *Hadena ectypa*.

Abstract

Background and Aims Population genetic structures and patterns of gene flow of interacting species provide important insights into the spatial scale of their interactions and the potential of local coadaptation. We analyzed the genetic structures of the plant *Silene stellata* and the nocturnal moth *Hadena ectypa*. *Hadena ectypa* acts as one of the pollinators of *S. stellata* as well as an obligate seed parasite. Although *H. ectypa* provides substantial pollination service to *S. stellata*, this system is largely considered parasitic due to the severe seed predation by the *Hadena* larvae. Previous research on this system has found variable interaction outcomes across space, indicating the potential for a geographical selection mosaic.

Methods Using eleven microsatellite markers for *S. stellata* and nine markers for *H. ectypa*, we analyzed the population genetic structure within and among three local populations. We also assessed the patterns and intensity of historical gene flow among populations.

Key Results We found no spatial genetic structure in the moth populations, while significant differentiation was detected among the local plant populations.

Conclusions Our results suggest that although the moths move frequently between populations, long-distance pollen carryover only happens occasionally. The difference in

gene flow rates between *S. stellata* and *H. ectypa* could potentially stabilize the interaction dynamics while at the same time prevent strict local coadaptation.

Introduction

Species interactions often occur at spatially discrete scales (Tilman and Kareiva 1997). Thus, spatial heterogeneity of biotic and abiotic factors among communities can result in differential ecological outcomes for these interactions, leading to a "geographic selection mosaic" (GSM) (Thompson 2005). Under the GSM framework, three low-scale phenomena including geographic selection mosaics, coevolutionary hotspots, and trait remixing lead to highly fluid ecological and genetic structures on the regional level, which in turn determine the broader coevolutionary dynamics on the evolutionary timescale including diversification and speciation (Thompson 2005).

Spatial genetic structures have strong effects on the coevolutionary dynamics of interacting species for the following reasons. First, if the fitness of a particular genotype of one of the interacting species depends on the genotypic distribution of its partner species, genetic differentiation between populations of the second species could result in differential interaction outcomes among populations. This interaction adds another dimension to the geographic selection mosaic and the resulting genotype-by-genotype-by-environment interaction introduces variability to local coevolutionary dynamics (Thompson 2005). Second, metapopulation processes including local extinction/recolonization and gene flow, strongly modify local interaction dynamics through the disruption of local genotypic distribution and can lead to coevolutionary outcomes substantially different from the dynamics of isolated populations. For example, a certain range of migration rates among populations helps stabilize host-parasite interactions in a metapopulation framework by reducing the extinction rates for the

parasite (Brown 1969; Hassell et al. 1991). Additionally, theoretical results demonstrate that, for a geographic selection mosaic consisting of two communities in which the pairwise interaction is antagonistic for one and mutualistic for the other, gene flow between the two demes could result in a global evolutionarily stable mutualism and high levels of local maladaptation and trait mismatching (Nuismer et al. 1999). Furthermore, the relative levels of gene flow of the two interacting species can also lead to divergent interaction outcomes (Gandon et al. 1996; Gandon and Michalakis 2002).

Plant vs. animal comparisons provides one of the sharpest contrasts between dispersal abilities of interacting species. Due to the sessile nature of plants we expect to observe stronger population genetic structure in plants especially those with biotic pollination and where seed dispersal is limited (Hamrick and Godt 1996). However, when the animal species is also a specialized pollinator of the plant, spatial genetic processes of the plant may largely be determined by the pattern of pollen flow mediated by behaviors of the pollinator (Brunet and Holmquist 2009). Pollinator behavior can affect population genetic structure both within the plant population (e.g., pollinators restricted to nearest-neighbor plants can increase the level of inbreeding) and among plant populations (e.g., through long-distance pollen dispersal). The potential gene flow asynchrony between a plant and its pollinator blurs the spatial scale of local coevolution and may have implications for plant-pollinator coevolution.

Here we examine the interaction between a native North American species pair: *Silene stellata* and *Hadena ectypa*, through a population genetic structure perspective. Nocturnal moths from the genus *Hadena* and plants of the Caryophyllaceae family, especially in the genus *Silene*, commonly form facultative interactions (Brantjes 1976*a*,

1976*b*; Kephart et al. 2006; Giménez-Benavides et al. 2007; Reynolds et al. 2012; Prieto-Benítez et al. 2016). The *Silene-Hadena* interactions in most species pairs have been considered parasitism (Brantjes 1976a, 1976b, Pettersson 1991, Bopp and Gottsberger 2004, Reynolds et al. 2012). Unlike the pollinators in more specialized interactions such as fig wasps and yucca moths, larvae of various *Hadena* species consume not only one fruit where they hatch, but also many more flowers and developing fruits before pupation, leading to a considerable fitness reduction for the plant (Reynolds et al. 2012). In addition to interactions with multiple species of *Hadena*, *Silene* species also represent specializations to different pollinator functional groups, coincident with their diverse floral display (Kephart et al. 2006). Closely related species often exhibit highly distinct floral designs, for example, three North American species *S. caroliniana, S. stellata and S. virginica* exhibit specialization to bee, moth and hummingbird pollination, respectively (Reynolds et al. 2009). The parasitic nature of the *Silene-Hadena* interaction and the great floral diversity in *Silene* beg a mechanistic explanation of how this interaction could persist in multiple lineages over evolutionary time and how transitions to and/or from nocturnal pollination had occurred.

The *Silene-Hadena* interaction is complex and both mutualism and parasitism occur at different life history stages of *H. ectypa*. Adult *H. ectypa* pollinate the hermaphroditic flowers of *S. stellata* while extracting nectar. In addition to pollination by the adult *H. ectypa*, oviposition by female moths inside the calyx following nectaring brings strong negative effects through larval predation on the reproductive tissues of *S. stellata* (Kula et al. 2013). This interaction is nonobligate in that although *H. ectypa* exclusively uses *S. stellata* as its host plant, *S. stellata* is also pollinated by a number of

generalist nocturnal moths (copollinators) that are equally effective in pollen transfer as *H. ectypa* (Reynolds et al. 2009; Kula et al. 2013). Depending on the relative densities of the copollinators, the net outcome of this interaction is spatially and temporally variable (Reynolds et al. 2012). Furthermore, previous research has shown a correlation between *S. stellata* floral traits and reproductive success (Reynolds 2008, Kula *et al.* 2013), corroborating the possibility of ongoing selection on floral traits by *H. ectypa* or mutual selection.

The goals of this study are to determine the population genetic structure within and among populations of *S. stellata* and *H. ectypa*, in three local populations within their native habitat in North America. Specifically, we quantify genetic diversity, fine-scale spatial genetic structures and migration patterns of these two closely interacting species to provide insight into the ecological structure and context dependency of this interaction on a metapopulation level.

Methods

Study species and their interaction

Silene stellata L. is an infrequent, iteroparous, long-lived perennial herb that is distributed throughout the eastern part of the United States. The plant flowers from early July through early September. It produces white, hermaphroditic, protandrous flowers (average of 25 ovules/pistil, Reynolds et al. 2009), which form panicle inflorescences. A plant usually produces multiple stems and on average produces ~40 flowers each flowering season (Reynolds et al. 2012). Outcrossing rate is relatively high (~73%,

Reynolds 2008; > 80%, Chapter 1). The flowers are pollinated by *Hadena ectypa* as well as by a number of generalist nocturnal moths that are equally effective at pollen transfer (Reynolds et al. 2009; Kula et al. 2013). The obligate seed predating pollinator *Hadena ectypa* is distributed from Massachusetts west to Minnesota and Kansas, and south to northern Georgia, concordant with the distribution of *S. stellata* (Schweitzer et al. 2011; Nelson 2012). Adult male and female *H. ectypa* nectar in the flowers of *S. stellata* with pollination taking place simultaneously. Oviposition behavior follows nectaring, as female moths lay single or multiple eggs at the base of the ovary or on the ovary wall (Zhou et al. 2016a). Forty percent of visits are followed by oviposition (Kula et al. 2013). The newly hatched larva bores into the ovary and develops through the third instar therein before starting to forage between flowers and plants. A larva can consume up to 40 flowers and/or unhardened fruits under lab conditions (Reynolds et al. 2012). A multi-year study conducted in the same sites investigated here documented fruit predation rates of 10-30% (Reynolds et al. 2012). The larvae pupate underground, and *H. ectypa* exhibits a univoltine life history at our study sites.

In contrast to the predominant diploidy of *Silene* species distributed in the Old World, the vast majority of North American *Silene* have been shown to be polyploids, including tetra-, hexa-, and octoploids, with tetraploidy's being the most common type (Popp and Oxelman 2007). It is unclear whether the lineage containing *S. stellata* was formed through autopolyploidization or autopolyploidization (Popp and Oxelman 2007). However, we observed maximum number of alleles per locus to be four across all 11 microsatellite loci, suggesting high homogeneity among chromosomes, which may indicate that *S. stellata* is a autotetraploid. The polyploidy introduces a number of

technical complications for the development of microsatellite markers and the downstream genotyping (Zhou et al. 2016b).

Study sites

We collected plant and animal tissues from three local habitats near Mountain Lake Biological Station in Giles County, Virginia, USA: Meadow (37.348296°, -80.544301°, elevation ≈ 1,100–1,300 m), Windrock (37.413889°, -80.519444°, elevation ≈ 1,300 m), Woodland (37.355415°, -80.553469°, elevation ≈ 1,100–1,300 m) (Fig. 1). The Meadow and Woodland sites are approximately 1.5 km apart, and both are isolated from the Windrock site by 7–8 km. In over 20 years of surveys we have not found any other populations of *S. stellata* defined by the triangle of the three sites, or nearby to any of the three sites. Among these three populations, Meadow has the largest population size of *S. stellata*, consisting of many 1000's of plants, while the Woodland site has a lower plant density and consists of several hundred individuals. Windrock is the smallest population, with only ~100 plants. These three sites also represent a wide range of habitats of *S. stellata*. The Meadow population is located on a powerline cut that is approximately 1 km long and 40 m wide. Woodland is a former chestnut forest and currently consists of mainly mature oak and hickory trees. Windrock is an exposed west-facing site with wind-topped oak trees.

Silene *tissue collection*

In the summer of 2011, we collected leaf tissues from 111 *S. stellata* plants. In the Meadow population, which had the highest plant density, a total of 40 plants were sampled along three transects with ~13 plants collected along each transect. Thirty-two

and 39 plants were collected along two transects in Windrock and Woodland, respectively, which have much smaller plant populations. Transects were linear and 50-300 m long. We sampled plants that were at least 5 m from each other to refrain from sampling the same plant as some plants produce multiple stems that can be up to a meter across, and also to sample the greatest genetic diversity within a population. From each plant, a single leaf was removed and stored in silica desiccant (Fisher Scientific). We recorded the GPS coordinates of the sampled plants. Genomic DNAs were extracted from 111 *S. stellata* individuals with an AutoGenprep 965/960 machine (AutoGen, Holliston, MA, USA) using the Plant DNA Extraction Kit AGP965/960 following the manufacturer's protocol.

Hadena collection and rearing

We sampled *H. ectypa* eggs in 2012 from the three sites by collecting *S. stellata* flowers along the same transects described above. To avoid sampling close relatives and to ensure a robust sample of within population genetic variation, we collected single flowers from plants that were spaced at least 10 m apart. Flowers were examined under a dissection scope for presence of *H. ectypa* eggs. Eggs were reared in petri dishes until the end of the third instar (about one week). The caterpillars were collected and stored in 100 percent ethanol. We kept no record of the exact locations where the larvae were collected, only from what site they were collected. To extract DNA, ~ 1g of larval tissue was placed in a 1.5ml centrifuge tube that was first frozen in liquid nitrogen, and then ground with a Micro Pestle. DNA was extracted from the ground tissue using the Qiagen Blood&Tissue kit (QIAGEN, Valencia, California, USA), following the manufacturer's protocol.

Isolation of microsatellite markers and genotyping

We collected genotypic data for eleven novel microsatellite loci designed for *S. stellata* with di- or trinucleotide repeats (Zhou *et al.* 2016). Genotypes of *H. ectypa* samples were obtained for a total of nine microsatellite loci. Eight loci previously designed for *H. bicuris* (Magalhaes et al. 2011) were tested in eight *H. ectypa* individuals. Of the eight tested, five loci (namely Hb02, Hb12, Hb19, Hb24, Hb29) produced bands consistently and were polymorphic. Additionally, we tested 72 candidate microsatellite loci designed based on Next-Generation Sequencing of one *H. ectypa* genome (unpubl.) and successfully identified and characterized four additional polymorphic loci (namely He58, He59, He62, He66).

DNA amplification was performed with fluorescently labeled forward primers (FAM- or HEX-) in 10-µL reactions, using the QIAGEN Type-it® Microsatellite PCR Kit. Each reaction contained the following components: ~10ng of genomic DNA, 5 µL of the 2x Multiplex PCR Master Mix, 1µL of the forward primer, and 1µL of the reverse primer (final concentration of the primers: 0.2µM). A touchdown PCR protocol was performed in a BIO-RAD T100 thermocycler (Bio-Rad, Hemel Hempstead, UK) using the following conditions: 95°C for 5 min; 5 cycles at 95°C for 30 s, 60°C for 1.5 min, and 72°C for 30 s; 28 cycles at 95°C for 30 s, 55°C for 1.5 min, and 72°C for 30 s; and a final extension at 60°C for 30 min. PCR products were diluted in nuclease-free water (dilutions ranged from 1:10 to 1:50), and 1 µL of each dilution was added to 9 µL of HiDi formamide with 1 µL ROX standard (DeWoody et al. 2004). Samples were heated to 95 °C for six minutes, cooled to 4 °C for six minutes, and loaded onto an ABI 3730xl automated capillary sequencer with a 50 cm, 96 channel array containing POP-7 polymer

for fragment analysis at the Laboratories of Analytical Biology (LAB) of the Smithsonian National Museum of Natural History. Fragment patterns were then visualized and scored using the GeneMapper V3.7 software (Applied Biosystems, Foster City, CA, USA). PCR and fragment profiling conditions were identical for *S. stellata* and *H. ectypa.*

Genetic diversity within populations

Levels of genetic diversity in the *S. stellata* and *H. ectypa* populations were evaluated by calculating the following statistics: the number of alleles per locus (A), effective number of alleles (A', number of alleles in a population weighted for their frequencies), observed heterozygosity (H_o) and expected heterozygosity (H_e).

Due to the tetraploidy of *S. stellata,* allelic dosage cannot be determined for partial heterozygotes (for example, an individual showing two alleles A, B on a given locus can have AAAB, AABB or ABBB as the underlying genotype). Hence we used the software Genodive version 2.0b27 (Meirmans and Van Tienderen 2004*b*) to characterize genetic diversity and pairwise differentiation between populations for both the tetraploid *S. stellata* and the diploid *H. ectypa,* to ensure consistency across the analyses. Genodive allows the unbiased estimations of allele frequencies and H_e within populations for data containing partial heterozygotes in a maximum likelihood framework under the assumption of random mating (De Silva et al. 2005).

We also calculated G_{IS} (Nei 1987) in each population for each locus as well as over all loci to evaluate the degree of inbreeding. In addition, each population was tested for departure from Hardy-Weinberg equilibrium (HWE) in each locus and across all loci. For the tetraploid *S. stellata,* H_o was calculated as gametic heterozygosity, i.e. 1.0 minus the probability that two random alleles drawn from the individual being the same:

AAAA=0, AAAB=0.50, AABB=0.66, AABC=0.83 and ABCD=1 (Bever and Felber 1992; Moody et al. 1993).

Population structure analysis and differentiation

We used the Bayesian model-based program STRUCTURE 2.3.4 (Pritchard et al. 2000) to test for the optimal number of genetic groups (denoted K hereafter) and estimated admixture proportions for each individual. To determine the optimal number of genetic clusters, we performed 20 independent runs for each K = 1–8 (Evanno et al. 2005), applying the admixture model in 20 replicates using a burn-in period of 10,000 followed by 10,000 steps. For better detection of subtle population structure given the relatively small spatial scale, we chose correlated allele frequencies among proposed clusters following the suggestion by Falush et al. (2003), and allowed the degree of admixture alpha to be inferred from the data. Given the adjacency of the study populations, we used sampling location as prior information by setting LOCPRIOR=1. The use of prior information has been suggested to be better at detecting weak divergence, while at the same time being unbiased toward spurious structures (Hubisz et al. 2009; Pritchard et al. 2010). The true K was determined using both estimates of the posterior probability of the data for a given K (Pritchard et al. 2000) and ΔK (Evanno et al. 2005).

We chose Hedrick's G'_{ST} (Hedrick 2005) as the measure of genetic differentiation between and among populations. F_{ST} and and its analogue G_{ST} are influenced by the degree of within-population polymorphism. Specifically, when large numbers of alleles are maintained within individual populations, both H_S and H_T can approach 1, even when different alleles are maintained in different populations, causing downwardly biased estimates of genetic differentiation (Hedrick 1999, 2005). Hedrick's G'_{ST} addresses this

challenge by scaling the observed value of differentiation by the maximum possible value given the level of polymorphism, and therefore allows the comparison of levels of genetic differentiation between *S. stellata* and *H. ectypa*, given the difference in allelic richness between these two species. We estimated single-locus and multi-locus G'_{ST} values and their significance with 9999 permutations in Genodive. A maximum likelihood method was utilized to correct for unknown dosage of the alleles for *S. stellata* (Meirmans 2013).

Migration patterns between populations

We used the coalescent-based program MIGRATE-N 3.6.11 (Beerli and Palczewski 2010) to test hypotheses about migration patterns and to estimate gene flow levels between populations. Five migration models were evaluated. All models contain three parameters of population sizes and differ in patterns of migration: 1) a full model with six directional migration rates; 2) a model with three symmetrical migration rates; 3) a model with two directional migration rates between Meadow and Woodland; 4) a model with one undirected migration rate between Meadow and Woodland (Windrock is considered an isolated population under models 3 and 4, given its relative isolation from Meadow and Woodland); 5) a null model consisting of only population sizes and no migration. We estimated the mutation-scaled effective sizes $\Theta=4N_e\mu$ (or $\Theta=8N_e\mu$ for the tetraploid *S. stellata*, Beerli 2012) , where N_e is the effective population size and μ is the mutation rate, as well as mutation-scaled migration rates $M = m/\mu$, where m is immigration rate per generation. We used Bayes factors for model comparison based on marginal likelihood values approximated with thermodynamic integration (Beerli 2012).

We used the Brownian motion mutation model with mutation rate estimated from the data for each locus. Relative mutation rates per locus scaled against the mean

mutation rate across loci ranged from 0.567 to 1.827 in *S. stellata*, from 0.444 to 2.111 for *H. ectypa*. For each locus, we performed parallel runs of 50 replicates, each consisting of a burn-in period of 100,000 MCMC steps, followed by 1,000,000 steps. Samples were recorded every 50 steps, resulting in a total of 20,000 recorded parameter values for each replicate. We used a heating scheme with 4 changes with temperatures 1.00, 1.50, 3.00, and 1000000.00, in order to improve the estimation of marginal likelihood (Beerli 2009). A random genealogy and parameter values inferred by an F_{ST} -based method were used as the initial condition for each chain. Prior distributions for Θ and and M were uniformly distributed with boundaries 0-200 and 0-5000, respectively.

Because MIGRATE-N only accepts diploid genotypes, we prepared pseudo-diploid data for *S. stellata.* In particular, we inferred the underlying genotypes for partial heterozygotes using the maximum likelihood procedure in Genodive (e.g. an individual with alleles A, B, and C on a locus was inferred as AABC, ABBC, or ABCC, depending on which configuration had the highest likelihood) (Meirmans 2013). We then converted the inferred tetraploid genotypes to pseudo-diploid genotypes by placing the four alleles of an individual on a locus on two separate data lines, following the instruction by Beerli (2012). Therefore, for each population, we had twice as many pseudo-diploid samples as the number of tetraploid individuals (Meadow: n=80; Windrock: n=64; Woodland: n=78). Parallel computation of MIGRATE-N was conducted using computational resources at the Maryland Advanced Research Computing Center (MARCC).

Results

<u>*Neutral genetic variation within populations*</u>

Silene stellata

We genotyped 111 individuals at 11 microsatellite loci. All loci were highly polymorphic with 342 alleles found over all loci (average number of alleles per locus = 31.1). Number of alleles per locus ranged from 16 (locus 3R and S12) to 55 (S44) (Supplemental Table S1). The population with the highest average number of alleles was Meadow (22.6), followed by Woodland (21.273), then by Windrock (18.5). We also observed a prevalence of private alleles in all loci and in all populations. The largest number of private alleles observed was 10 (locus S44 in Windrock). Additionally, a large number of rare alleles were found to be common: over all loci, 141 alleles (41.2%) were present at a frequency <2% among all populations.

High single-locus H_e was observed in all three populations, ranging between 0.599 (S12 in Meadow) and 0.953 (S71 in Meadow) (Supplemental Table S1). Over all loci, Windrock had the highest $H_e = 0.879$, followed by Woodland ($H_e = 0.870$) and Meadow ($H_e = 0.864$) (Table 1). Although we observed significant G_{IS} scores in some population-by-locus combinations (Supplemental Table S1), multi-locus G_{IS} in all three populations were positive, but not significant (Table 1). Note that due to the technical difficulty caused by partial heterozygotes, the H_o's were not estimated based on unbiased gametic heterozygosity rates and assessments of G_{IS} and HWE were conservative.

Hadena ectypa

From the 96 *H. ectypa* individuals genotyped for nine microsatellite loci, 84 alleles were found over all loci and populations. Average number of alleles per locus was much lower than *S. stellata* (9.333 in *H. ectypa,* compared with 31.091 in *S. stellata*). Across all populations, Hb12 had the lowest number of alleles (3) and He59 had the highest (20) (Supplemental Table S2). The population with the highest average number of alleles was Woodland (7.222), followed by Meadow (6.333) and Windrock (6). The observed heterozygosity averaged over all loci ranged between 0.326 and 0.373 (Table 1). With few exceptions, we observed strong heterozygote deficit in all loci and in all three populations. Additionally, most of the population-by-locus combinations showed significant deviation from HWE (Supplemental Table S2). Over all loci, all three populations presented significant deviation from HWE ($p < 0.001$), with inbreeding coefficients ranging from 0.341 (Windrock) to 0.440 (Woodland) (Table 1).

<u>*Neutral genetic divergence between populations*</u>

Silene stellata

We found a significant level of global genetic differentiation, $G'_{ST} = 0.09$ ($p < 0.001$). Pairwise G'_{ST} were significant between Meadow and Windrock ($G'_{ST} = 0.146$, $p < 0.005$), Woodland and Windrock ($G'_{ST} = 0.215$, $p < 0.005$), but only trended towards significant between Meadow and Woodland ($G'_{ST} = 0.081$, $p = 0.087$) (Table 2).

We estimated optimal number of genetic clusters using STRUCTURE for the *S. stellata* samples as K=2, which had the highest posterior probability ($p > 0.999$). The ΔK criterion supported the existence of six distinct genetic groups (Figure 2). For K=2, The

similarity between Meadow and Woodland and their divergence from Windrock are revealed by their population admixture proportions: Meadow (0.089, 0.911); Woodland (0.086, 0.914); Windrock (0.666, 0.334) (Figure 2A).

Hadena ectypa

Neutral genetic divergence among the three studied populations was very low and not significant. Pairwise G'_{ST} ranged between 0.001 (Meadow vs. Windrock) and 0.005 (Windrock vs. Woodland) with overall $G'_{ST} = 0.003$ ($p = 0.270$). The single locus global G'_{ST} ranged from 0.000 to 0.038 (Table 2).

Contrary to the insignificant G'_{ST}, both posterior probabilities of the data and ΔK identified K=3 as the highest probable genetic structure between the three sample populations (estimated ln probability of data = -1896.0, $p > 0.999$) (Figure 3).

<u>*Historical gene flow*</u>

Silene stellata

Bayes factors calculated as the ratio of likelihoods between two competing models strongly supported the full model with directional gene flow between all populations against the four alternative models (Bayes Factor K > 100 for all comparisons between the full model and alternative models, Kass and Raftery 1995). Among the three populations, Woodland had the largest effective population size estimated using the Bayesian approach ($\Theta = 8N_e\mu = 3.575$), followed by Meadow ($\Theta = 2.551$), with Windrock being the smallest ($\Theta = 1.377$) (Table 3; Figure 4)

Long-term migration rate (scaled by mutation M = m/μ) estimated using the Bayesian approach was variable and asymmetrical among populations. The highest migration rate was from Woodland to Meadow (M = 42.470), which was almost ten times stronger than from Meadow to Windrock (M = 5.759) (Table 3; Figure 4).

Hadena ectypa

Bayes factors also supported the full migration model for *H. ectypa*. Similar to *S. stellata*, the Woodland population was identified as having the largest effective population size ($\Theta = 4N_e\mu = 2.861$), followed by Meadow ($\Theta = 1.371$), with Windrock as the smallest ($\Theta = 1.209$) (Table 3; Figure 5). Migration rates between populations of *H. ectypa* were more uniform than *S. stellata*, with M ranging between 34.336 (from Windrock to Meadow) and 65.220 (from Woodland to Windrock) (Table 3; Figure 5). Under the assumption of similar mutation rates between the two species, mean gene flow rate per generation in *H. ectypa* was twice as large as *S. stellata*: $m_{He} / m_{Ss} = (\mu_{He} / \mu_{Ss}) \times 2.2$, while mean effective population sizes were similar $4N_{He} / 8N_{Ss} = (\mu_{Ss} / \mu_{He}) \times 0.7$.

Discussion

Unlike fig wasps and yucca moths, which form obligate mutualism with their host plants, the *Silene-Hadena* interactions range from parasitic to potentially mutualistic (Kephart et al. 2006), dependent upon a suite of ecological factors influencing the ecological outcome and coevolutionary trajectory of this interaction. Among these factors, discordance in the

population genetic structures and patterns gene flows are of great importance in that they do not just provide the spatial background for this interaction but could also actively determine the coevolutionary outcome (Gandon et al. 1996; Nuismer et al. 1999). Here, we studied the genetic structures of *Silene stellata* and *H. ectypa* in order to understand the potential for local coadaptation and the consequences for the maintenance of this interaction.

Within population genetic diversity

For *S. stellata,* all loci showed very high levels of variability and rare alleles were also very common. A number of studies utilizing microsatellites in polyploid plants have found similarly high levels of rare alleles and allelic richness (Truong et al. 2007; Donkpegan et al. 2015). Rare alleles in tetraploid genomes have been suggested to be lost at a much slower rate than in diploids and that tetraploids often exhibit higher polymorphism than related diploids (Bever and Felber 1992). This can be partly understood to be the consequence of the increased effective population size caused by the doubling of individual genomes, which alleviates the effect genetic drift and allows higher neutral polymorphism.

The unknown dosage of alleles for partial heterozygotes in polyploids makes it challenging to calculate allele frequencies and other summary statistics dependent on allele frequency estimation, such as expected heterozygosity and fixation index. We estimated population-level allele frequencies using the iterative method of Genodive which maximizes the likelihood of the observed allelic phenotypes (Meirmans 2013). This procedure is a modification of the expectation-maximization method introduced by De Silva et al. (2005) and represents one of the state-of-the-art methods allowing the

correction of unknown allelic dosage (Dufresne et al. 2014). While certain biases are introduced by the the assumption of random mating, the high allelic richness and high proportion of full heterozygotes across loci should make our estimation of H_e, as well as other allele frequency-based statistics including G'_{ST} and gene flow rates relatively robust.

Because of allelic dosage uncertainty, we cannot directly assess gametic heterozygosity for partial heterozygotes (for example for a phenotype AB at a given locus, the underlying genotypes ABBB and AABB have gametic heterozygosity of 0.5 and 0.66). Therefore, our estimate of observed heterozygosity and inbreeding for *S. stellata* could only be based on the frequency of full homozygotes. This makes our estimates of H_o , G_{IS} as well as the test of HWE conservative for *S. stellata*, since full homozygosity rate in polyploids is less sensitive to inbreeding than in diploids (Bever and Felber 1992). In light of this technical difficulty inherent to tetraploidy, our estimates of H_o and G_{IS} did not show significant inbreeding within the three populations of *S. stellata.*

A previous fluorescent-dye study showed pollen dispersal distance (mean ± SE) of *S. stellata* to be 1.2 ± 0.35 m by diurnal pollinators and 2.2 ± 0.43 m by nocturnal pollinators (Reynolds et al. 2009), while in chapter 1 I demonstrate that most pollen dispersal is less than 3 m. Furthermore *H. ectypa* pollinators of *S. stellata* plants isolated by 30 m from the main Meadow population deposit very little pollen, suggesting that pollen dispersal between populations will be very low (Kula et al. 2014). Additionally, seeds of *S. stellata* primarily disperse passively through gravity. In other plant species, limited pollen and seed dispersal ability have been known to increase inbreeding (Fenster

1991*a*, 1991*c*; Richards 2000; Herlihy and Eckert 2002; Fenster et al. 2003; Whelan et al. 2009). Other than the possibility that significant inbreeding is masked by the biased estimate of HWE, other factors could counterbalance the effect of short pollen and seed dispersal distances. First, although pollen dispersal distance is short, the probability that a *S. stellata* individual receives pollen from a single source plant is low (0.05-0.12, Reynolds 2008), possibly reducing the chance of bi-parental inbreeding. Additionally, a relatively high outcrossing rate of ~73% (Reynolds 2008) in our *S. stellata* populations could counteract the effect of short pollen/seed dispersal distance.

In contrast to *S. stellata*, we found high levels of inbreeding in *H. ectypa*, with significant multi-locus G_{IS} values ranging from 0.341 to 0.440, although *H. ectypa* seems to have a high level of gene flow suggested by the observed weak genetic differentiation. Similar levels of significant inbreeding coefficients were quantified in a microsatellite based study of the European species *Silene latifolia* and *Hadena bicuris* (Magalhaes et al. 2011). These results suggests that there may be common or shared aspects of *Hadena* behavior responsible for the high observed inbreeding coefficients across species. In the flowering season of *S. stellata*, female *H. ectypa* often lay multiple eggs within the same flower (Zhou et al 2016). Additionally, the short pollen dispersal distance suggests female moths could also lay eggs in adjacent flowers and/or plants. The clustered egg laying behavior could potentially cause mating between siblings given the limited movement of larvae and if the moths mate immediately after their emergence. Subsequent long-distance dispersal by adults, but especially females, could then result in the lack of population genetic differentiation coupled with strong inbreeding. Another

scenario that could produce the empirical result detected would be if there were few adults mating and laying eggs, which could lead to inbreeding, followed by dispersal.

Population genetic structure

We found a significant global genetic differentiation, noted by the G'_{ST} values of the three *S. stellata* populations consistent with other studies of outcrossing herbaceous plants (Hamrick and Godt 1996). Pairwise G'_{ST} values were significant for Windrock vs. Meadow and Windrock vs. Woodland, but not significant between Meadow and Woodland. This is not surprising, as Windrock is the most remote population, separated from the other two populations by ~8 km, whereas the distance between Meadow and Woodland is ~1.5 km. Therefore, although the number of sampled populations is small, the observed G'_{ST} values do seem to support isolation by distance at the between population level.

Bayesian clustering results of *S. stellata* supported the existence of two or six genetic groups, based on different model selection criteria. Cryptic structures within populations could potentially explain a cluster number higher than the actual number of sampled populations. The similarity between Meadow and Woodland and their divergence from Windrock are made obvious by their population admixture proportions for K=2 (Figure 2A).

Both the G'_{ST} and STRUCTURE results indicate a gene flow barrier existing between Windrock and the other two populations. In a study of the European species *Silene latfolia*, significant differentiation ranging from 0.05-0.2 was observed between subpopulations separated by only a few hundred meters and up to 2 km (Barluenga et al. 2010). However, we found the G_{ST} between Meadow and Woodland to be only about one

tenth the magnitude of *S. latifolia* under similar isolation and not significant, although these two populations are separated by ~2 km. This low G_{ST} should not be be an issue of statistical power since we sampled twice as many plants per *S. stellata* population as the aforementioned cited study. One possible explanation is that Meadow and Woodland were originally connected but recently separated by human induced fragmentation. Alternatively, it could imply that genetic differentiation in *S. stellata* occurs on a larger spatial scale than *S. latfolia.* Differences in life history of these two species could potentially explain this discrepancy. *S. latifolia* is a short-lived perennial that mainly grows in disturbed habitats where demographical fluctuation and even local extinction could be prevalent (Richards et al. 2003), whereas *S. stellata* is a long-lived perennial. The potentially larger effective population size resulting from the longevity as well as the polyploidy of *S. stellata* could better buffer the effect of genetic drift and result in less genetic differentiation between populations, even under similar levels of gene flow. Tetraploids have half the decrease in rate of loss of heterozygosity (Bever and Felber 1992; Falconer and Mackay 1996), which could also contribute to the difference in genetic differentiation between these two species given recent population subdivision.

In contrast, we observed insignificant genetic differentiation between the three populations of *H. ectypa* based on the G'_{ST} estimation. For the STRUCTURE result, although a cluster number K=3 was best supported, the population admixture proportions were very similar across all three populations (Figure 3A), suggesting low genetic differentiation between populations. Sampling locations of *H. ectypa* individuals were used as priors in STRUCTURE. The use of prior information has been suggested to be better at detecting weak divergence with less data, while at the same time being unbiased

toward spurious structures. Therefore, the discrepancy between G'_{ST} values and STRUCTURE results could potentially be attributed to the difference in sensitivity of these two measures. The high observed inbreeding of *H. ectypa* presented a violation of the assumption of HWE by STRUCTURE and could potentially lead to a biased estimation of K. Alternatively, cryptic structure within populations caused by mating between relatives (as well as assortative mating) could also lead to genetic structuring not detectable at the population level. In any case, we believe the 10-100 fold difference in the G-statistics suggests gene flow among *H. ectypa* populations occurs much more frequently than among *S. stellata* populations, and that, although high *H. ectypa* movement was observed among all three populations, the ~8 km isolation between Windrock and the other two populations creates a barrier for *Silene stellata* pollen dispersal. Finally, the detection of a higher frequency of private alleles within the *S. stellata* populations in contrast to the frequency of private alleles detected from the sampling of *H. ectypa* eggs corroborates the different gene flow patterns of the two species across population genetic scales.

Consequences for Geographic Selection Mosaic

The gene flow asynchrony between the two species may have important implications for the coevolutionary dynamics of the *Silene-Hadena* interaction. First, the significant genetic structure and the restricted gene flow over long distances of *S. stellata* provide the potential for local adaptation of floral traits involved in the interaction with *H. ectypa*. However, the high gene flow rate and the lack of genetic structure of local *H. ectypa* populations may prevent strict local coadaptation. Secondly, this discrepancy between migration abilities could also have a stabilizing effect. Theoretical work has shown that

when gene flow of the parasite is stronger than the host, the parasite populations are selected for adaptation to local host types while the host are selected to be resistant to all parasite types, promoting trait polymorphism within local host populations (Gandon et al. 1996). High parasite gene flow brings in non-locally adapted genotypes, which in turn increases local host resistance. This increased host resistance could potentially alleviate the negative parasitic effect and stabilize the interaction dynamics.

While previous work conducted in the same populations indicated the possibility of a geographical selection mosaic (Reynolds et al. 2012), the spatial asynchrony of gene flow between *S. stellata* and its pollinating seed parasite *H. ectypa* implies that the coevolutionary dynamics could be determined by the complex interplay between local processes favoring the escape of *S. stellata* from this interaction and the stabilizing effect of spatial processes on the metapopulation level. Similar gene flow asynchrony in the European *S. latifolia – H. bicuris* system was observed in a study area across central Europe (Magalhaes et al. 2011). The recurrent pattern observed on different spatial scales, together with the distinct life history strategies and ploidy levels of *S. stellata* and *S. latfolia*, suggest gene flow asynchrony could be a robust mechanism influencing the evolution and maintenance of the *Silene-Hadena* interaction between various lineages from the two genera.

Table 1. Genetic diversities of *Silene stellata* and *Hadena ectypa* in three local populations and across populations near Mountain Lake Biological Station. Results were based on 11 microsatellite loci for *S. stellata* and 9 microsatellite loci for *H. ectypa*. N=number of individual sampled; *A* = number of alleles; *A'*= effective number of alleles; H_o = observed heterozygosity; H_e = expected heterozygosity; G_{IS} = inbreeding coefficient.

	S. stellata						*H. ectypa*					
Population	*N*	*A*	*A'*	H_o	H_e	G_{IS}	*N*	*A*	*A'*	H_o	H_e	G_{IS}
Meadow	40	22.636	9.196	0.765	0.864	0.115ns	32	6.333	2.793	0.358	0.554	0.354***
Windrock	32	18.455	8.768	0.698	0.879	0.206ns	31	6	2.892	0.373	0.567	0.341***
Woodland	39	21.273	8.582	0.770	0.870	0.115ns	34	7.222	3.080	0.326	0.582	0.440***
Overall	111	31.091	8.814	0.757	0.887	0.146ns	98	9.333	2.884	0.352	0.569	0.379***

*** $P<0.001$; ns, not significant.

Table 2. Pairwise and global *G*-statistics (G'_{ST}, Hedrick 2005) between three local populations of *Silene stellata* and *Hadena ectypa*. Results were based on 11 microsatellite loci for *S. stellata* and 9 microsatellite loci for *H. ectypa*. M = Meadow; R = Windrock; W = Woodland.

	Silene stellata Global G'_{ST}=0.09, p <0.001			***Hadena ectypa*** Global G'_{ST}=0.005, p > 0.10		
	M	R	W	M	R	W
M	-	**	**	-	ns	ns
R	0.146	-	ns	-0.002	-	ns
W	0.081	0.215	-	0.008	0.008	-

Note: G'_{ST} values in the lower matrix and significance (*** P<0.001; ** P<0.01; *P<0.05; ns, not significant) in the upper matrix.

Table 3. Mean and 95% confidence intervals of mutation scaled population sizes ($\Theta = 8N_e\mu$ for *Silene stellata,* $\Theta = 4N_e\mu$ for *Hadena ectypa*) and directional migration rates ($M = m/\mu$) between three local populations of *S. stellata* and *H. ectypa* based on Bayesian inference of effective sizes and migration rates using MIGRATE-N 3.2.6. M = Meadow; R = Windrock; W = Woodland.

	S. stellata		*H. ectypa*	
Parameter	Mean	95% CI	Mean	95% CI
Θ_M	2.551	(0.000, 5.733)	1.371	(0.000, 4.667)
Θ_R	1.377	(0.000, 4.667)	1.209	(0.000, 4.400)
Θ_L	3.575	(0.000, 7.067)	2.861	(0.000, 6.267)
$\mathbf{M_{R\text{->}M}}$	14.889	(16.300, 20.133)	34.336	(0.000, 120.000)
$\mathbf{M_{W\text{->}M}}$	42.470	(33.730, 49.070)	40.094	(0.000, 126.667)
$\mathbf{M_{M\text{->}R}}$	5.759	(2.667, 6.500)	52.393	(0.000, 136.667)
$\mathbf{M_{W\text{->}R}}$	15.930	(13.800, 17.730)	65.220	(0.000, 146.667)
$\mathbf{M_{M\text{->}W}}$	25.530	(19.730, 31.200)	47.889	(0.000, 130.000)
$\mathbf{M_{R\text{->}W}}$	26.890	(18.400, 33.470)	47.200	(0.000, 130.000)

Figure 1. Geographical locations of three *Silene stellata* and *Hadena ectypa* study populations. GPS coordinates: Meadow, 37.348296°, -80.544301°; Windrock, 37.413889°, -80.519444°; Woodland, 37.355415°, -80.553469°.

Figure 2A. Estimated population structure of *Silene stellata*, for K=2 and K=6, based on genotypes of 111 *S. stellata* individuals on 11 microsatellite loci. Individuals are represented by vertical bars representing estimated genomic proportions corresponding to the proposed K. Figure 2B. Mean posterior probabilities of 20 independent runs for each K, K=1 to K=8. Figure 2C. ΔK for K=2 to K=7. K=2 had the highest peak height (Evanno et al. 2005).

Figure 3A. Estimated population structure of *Hadena ectypa*, for K=3, based on genotypes of 95 *H. ectypa* individuals on nine microsatellite loci. Individuals are represented by vertical bars representing estimated genomic proportions corresponding to three genetic clusters. Figure 3B. Mean posterior probabilities of 20 independent runs for K=1 to K=8. Figure 3C. ΔK for K=2 to K=7. K=3 had the highest peak height (Evanno et al. 2005).

Figure 4. Patterns of historical gene flow between three local populations of *Silene stellata* near Mountain Biological Station in Giles County, Virginia, based on MIGRATE-N 3.2.6 using 11 microsatellite markers. Six directional migration rates ($M = m/\mu$) are shown together with three mutation scaled effective population sizes ($\Theta = 8N_e\mu$).

Figure 5. Patterns of historical gene flow between three local populations of *Hadena ectypa* near Mountain Biological Station in Giles County, Virginia, based on MIGRATE-

N 3.2.6 using 9 microsatellite markers. Six directional migration rates ($M = m/\mu$) are shown together with three mutation scaled effective population sizes ($\Theta = 4N_e\mu$).

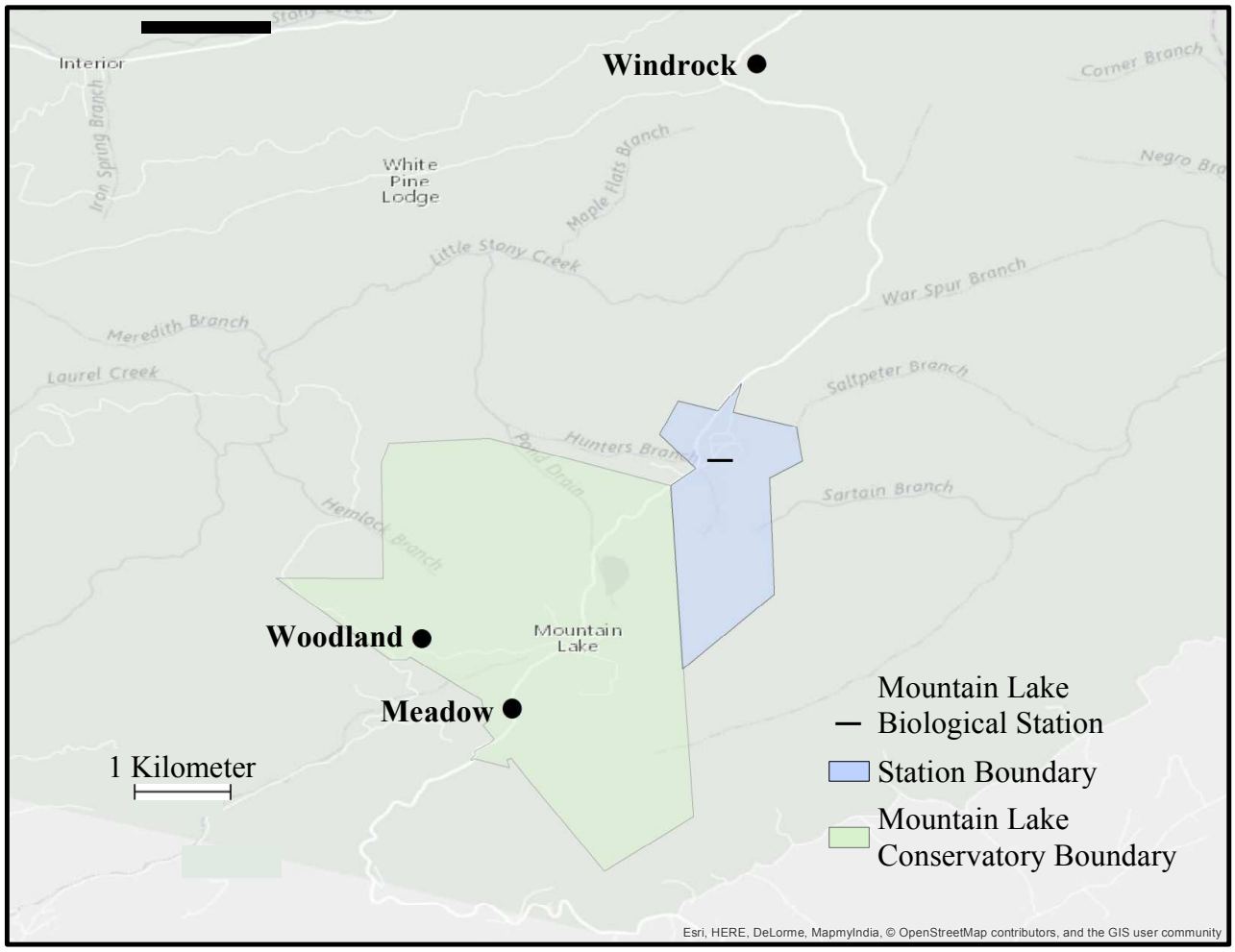

Windrock
Woodland
Meadow
Mountain Lake
Interior
White Pine Lodge
Iron Spring Branch
Maple Flats Branch
Little Stony Creek
Meredith Branch
Laurel Creek
Hunters Branch
Pond Drain
Hemlock Branch
Corner Branch
Negro Bra
War Spur Branch
Saltpeter Branch
Sartain Branch
1 Kilometer
Mountain Lake Biological Station
Station Boundary
Mountain Lake Conservatory Boundary
Esri, HERE, DeLorme, MapmyIndia, © OpenStreetMap contributors, and the GIS user community

FIG 1.

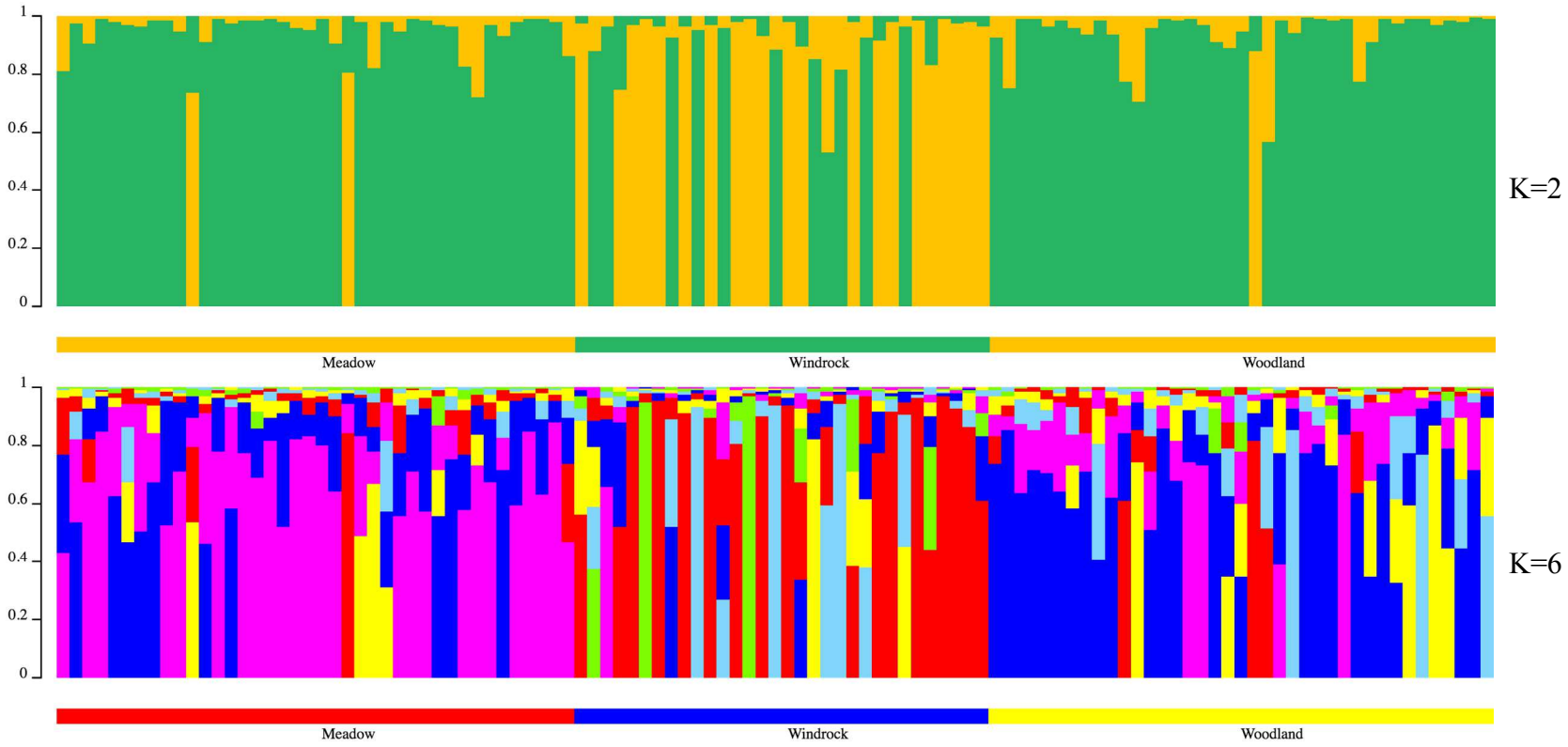

1
0.8
0.6
0.4
0.2
0
K=2
Meadow
Windrock
Woodland
1
0.8
0.6
0.4
0.2
0
K=6
Meadow
Windrock
Woodland

FIG. 2A

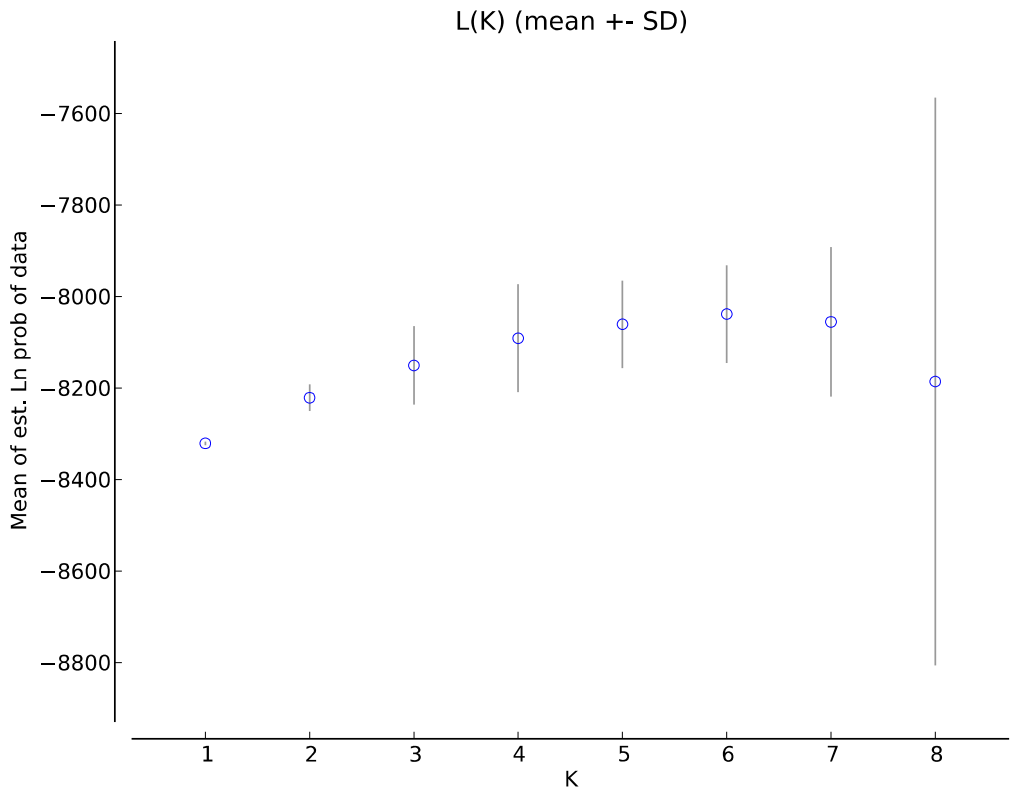

L(K) (mean +- SD)
Mean of est. Ln prob of data
−7600
−7800
−8000
−8200
−8400
−8600
−8800
1
2
3
4
5
6
7
8
K

FIG. 2B

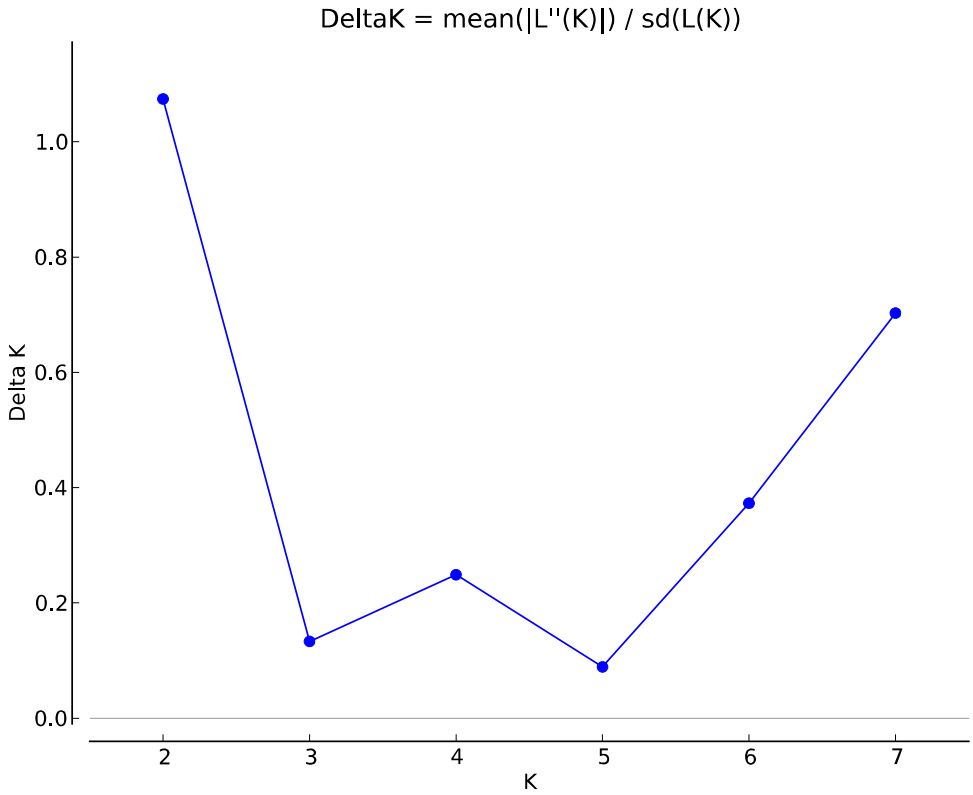
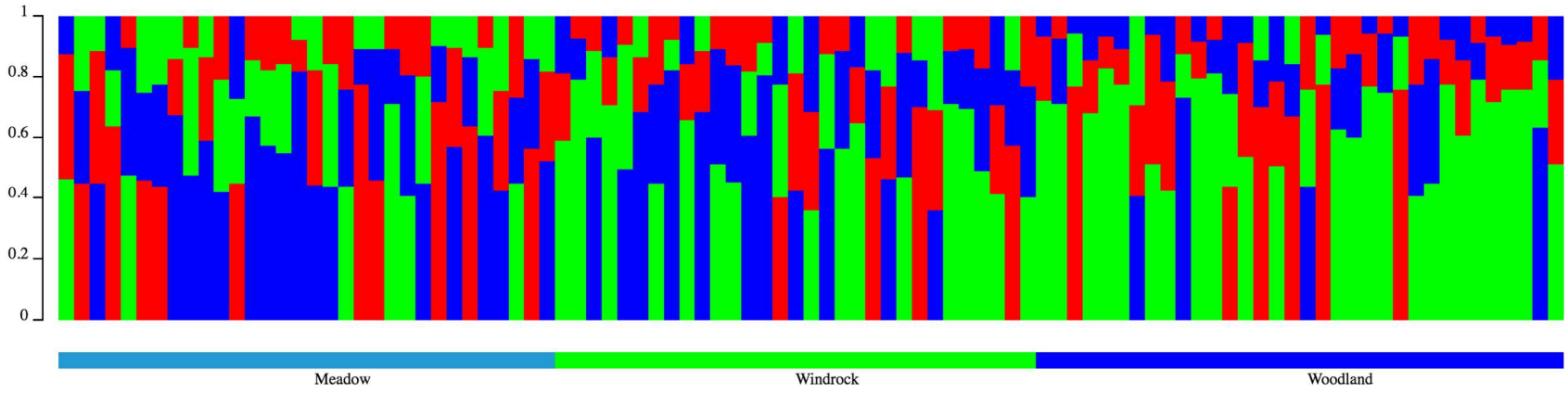


FIG. 2C



K=3

FIG. 3A

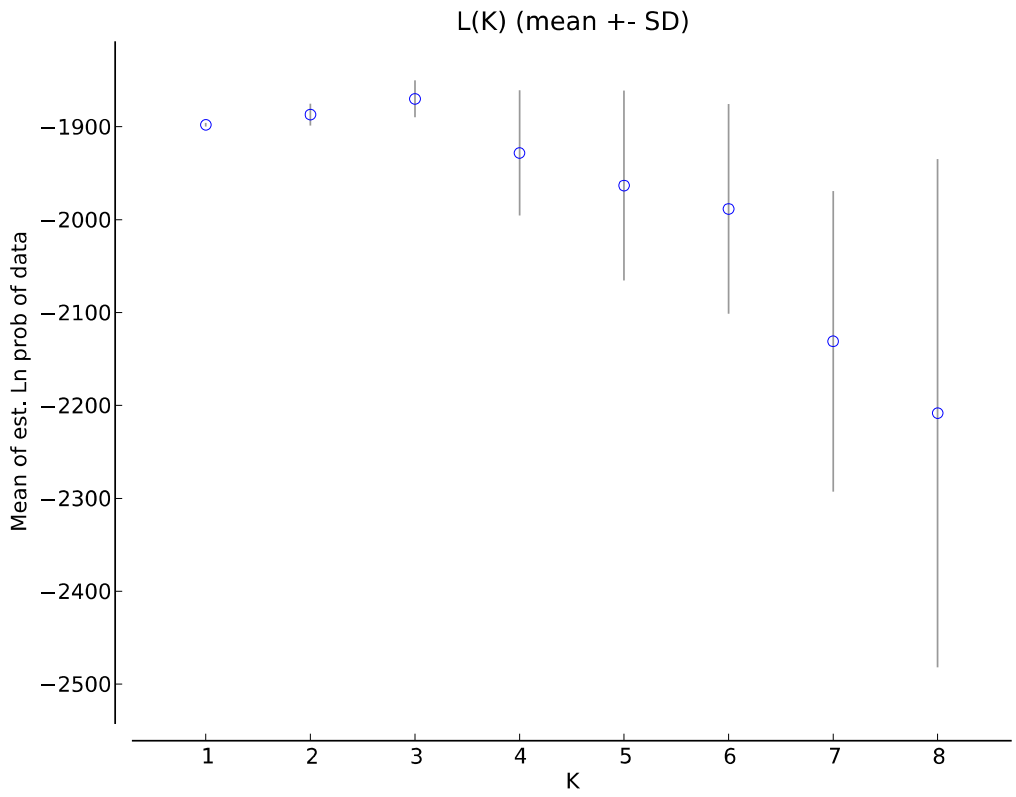

L(K) (mean +- SD)
Mean of est. Ln prob of data
−1900
−2000
−2100
−2200
−2300
−2400
−2500
1
2
3
4
5
6
7
8
K

FIG. 3B

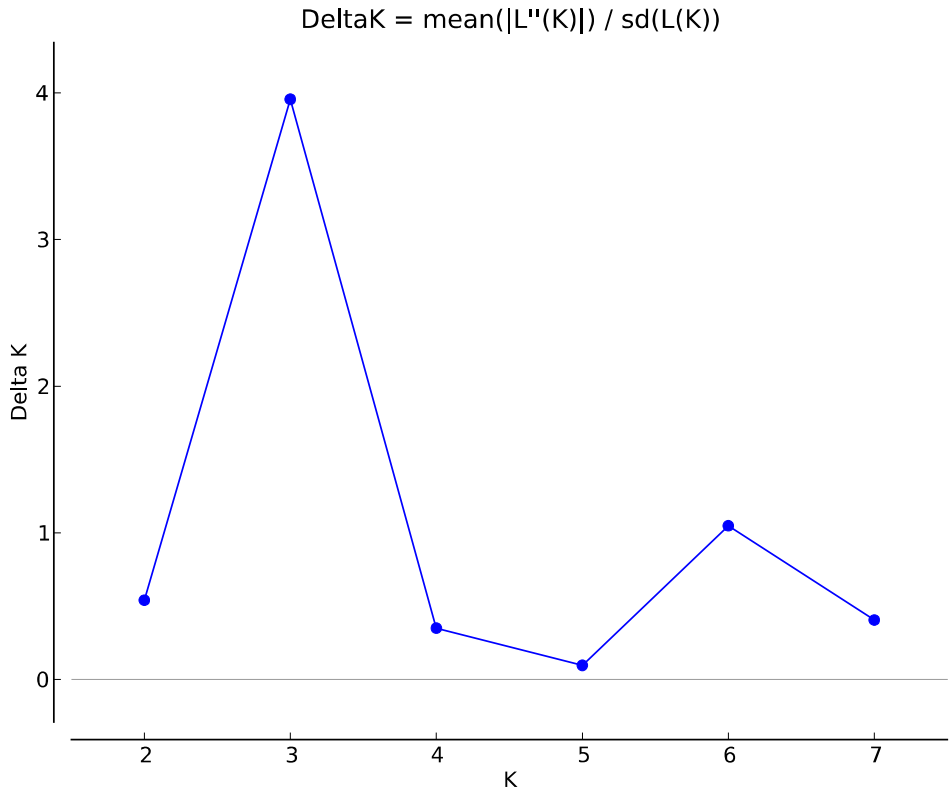


FIG. 3C

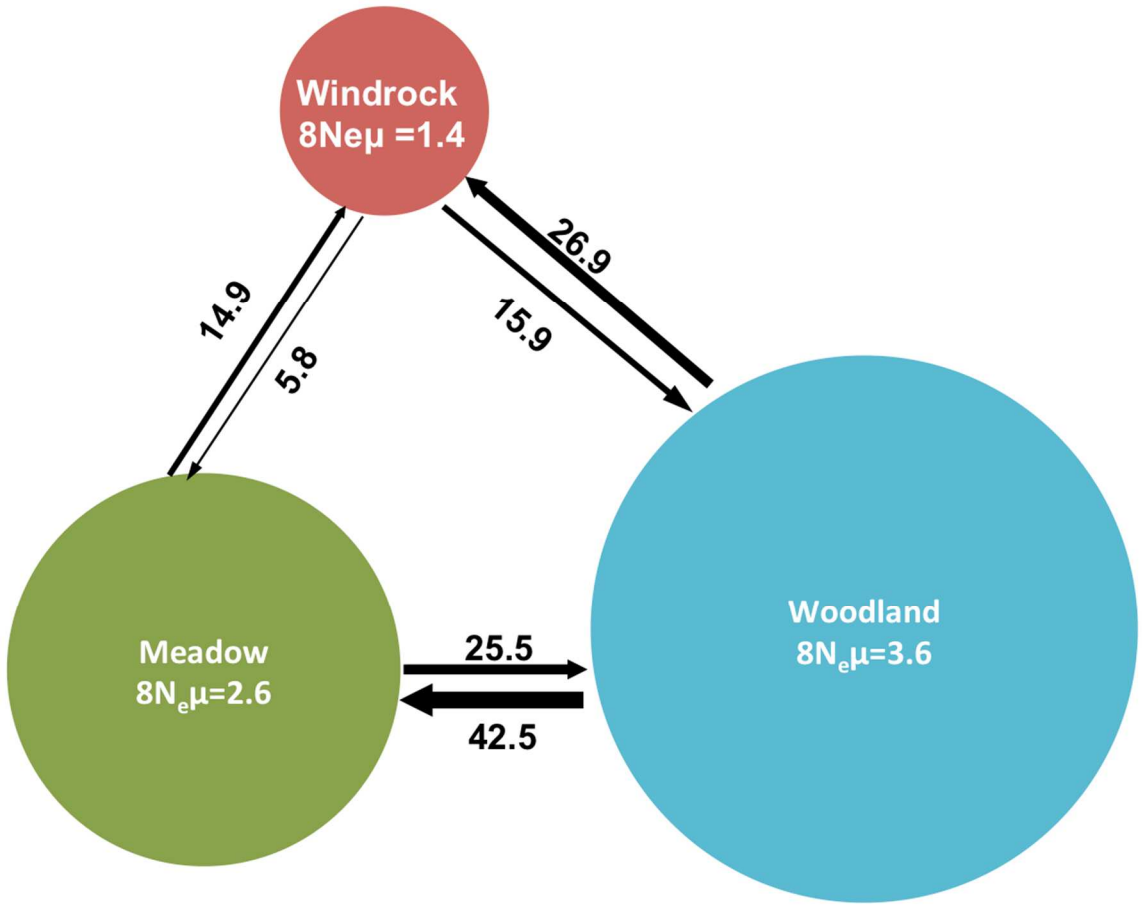

Windrock
8Neμ =1.4
14.9
5.8
26.9
15.9
Meadow
8Neμ=2.6
25.5
42.5
Woodland
8Neμ=3.6

FIG. 4

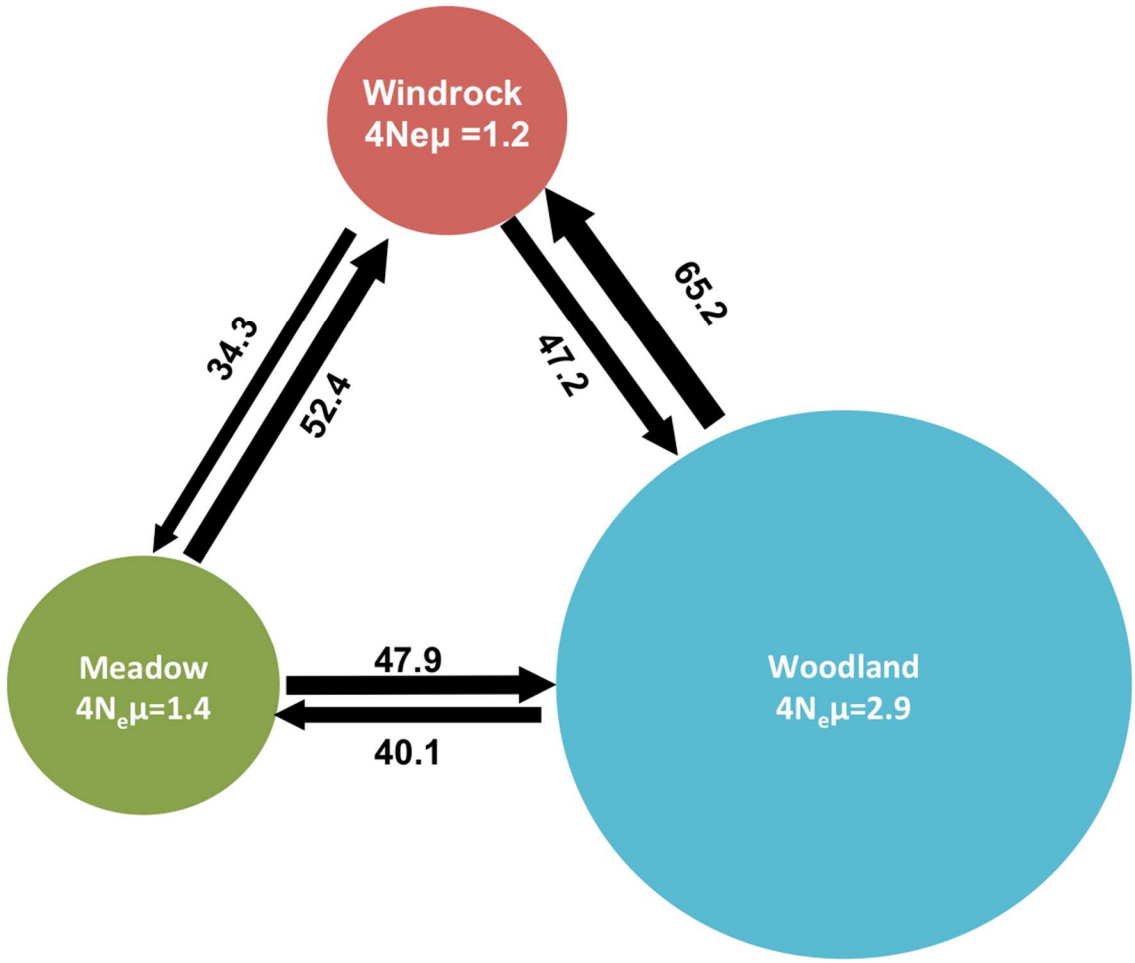

Windrock
4Neμ =1.2
34.3
52.4
47.2
65.2
Meadow
4Neμ=1.4
47.9
40.1
Woodland
4Neμ=2.9

FIG. 5

Supplemental Table S1. Genetic diversities of 11 polymorphic microsatellites of *Silene stellata*.

	Meadow (n=40)					Windrock (n=32)					Woodland (n=39)					Total (n=111)		
Locus	*A*	*Ap*	H_o	H_e	G_{IS}	*A*	*Ap*	H_o	H_e	G_{IS}	*A*	*Ap*	H_o	H_e	G_{IS}	*A*	H_o	H_e
1E	28	4	0.905	0.884	-0.024**	20	3	0.720	0.878	0.185	29	3	0.809	0.909	0.110	36	0.812	0.898
S10	12	1	0.572	0.826	0.308	11	2	0.449	0.800	0.456	14	5	0.461	0.868	0.469*	20	0.494	0.847
3R	14	0	0.984	0.883	-0.114***	14	2	0.976	0.881	-0.105	14	0	0.941	0.869	-0.083***	16	0.967	0.878
S12	6	1	0.349	0.599	0.418	11	3	0.243	0.800	0.594	12	4	0.484	0.642	0.246	16	0.359	0.694
3S	19	3	0.808	0.869	0.070	18	3	0.665	0.921	0.235	25	7	0.747	0.925	0.192	32	0.740	0.928
S44	34	9	0.900	0.890	-0.011**	32	10	0.936	0.898	-0.052	33	6	0.883	0.887	0.004	55	0.907	0.901
S23	25	3	0.986	0.916	-0.076***	22	2	0.998	0.905	-0.090	21	2	0.957	0.895	-0.069***	27	0.980	0.909
S71	30	4	0.934	0.953	0.020	23	2	0.858	0.936	0.100	30	4	0.902	0.945	0.046	38	0.898	0.959
2G	30	6	0.885	0.946	0.064	20	2	0.829	0.951	0.124	23	2	0.866	0.909	0.047	36	0.860	0.954
3I	32	9	0.775	0.915	0.153	19	3	0.559	0.927	0.389	18	1	0.738	0.838	0.119	36	0.691	0.915
3J	19	7	0.334	0.830	0.597**	13	4	0.399	0.791	0.519	15	5	0.684	0.882	0.224	30	0.473	0.858
Average	22.636	4.273	0.766	0.865	0.115	18.455	3.273	0.687	0.881	0.206	21.273	3.545	0.770	0.870	0.115	31.091	0.741	0.885

Note: A = number of alleles; A_p = number of private alleles; H_o = observed heterozygosity; H_e = expected heterozygosity; n = number of individuals sampled; G_{IS} = inbreeding coefficient. *** $P<0.001$; ** $P<0.01$; *$P<0.05$; ns, not significant.

Supplemental Table S2. Genetic diversities of 9 polymorphic microsatellites of *Hadena ectypa*.

	Meadow (n=32)					Windrock (n=31)					Woodland (n=33)					Total (n=96)		
Locus	*A*	*Ap*	H_o	H_e	G_{IS}	*A*	*Ap*	H_o	H_e	G_{IS}	*A*	*Ap*	H_o	H_e	G_{IS}	*A*	H_o	H_e
Hb02	6	0	0.594	0.541	-0.097	5	0	0.464	0.546	0.150**	7	1	0.452	0.594	0.239**	7	0.503	0.557
Hb12	3	0	0.233	0.264	0.117**	2	0	0.423	0.449	0.058**	3	0	0.226	0.303	0.255**	3	0.294	0.348
Hb19	8	2	0.500	0.778	0.357***	5	3	0.267	0.774	0.656***	9	3	0.414	0.784	0.473***	11	0.393	0.775
Hb24	3	0	0.103	0.196	0.472***	3	0	0.080	0.154	0.481***	4	0	0.107	0.234	0.731***	4	0.097	0.193
Hb29	11	5	0.241	0.778	0.690***	10	3	0.391	0.794	0.507***	10	2	0.417	0.833	0.500***	18	0.350	0.799
He58	3	0	0.222	0.204	-0.087	3	2	0.148	0.142	-0.045	4	2	0.172	0.224	0.229***	5	0.181	0.192
He59	11	3	0.633	0.762	0.169**	13	1	0.652	0.819	0.204***	15	4	0.517	0.765	0.324***	20	0.601	0.780
He62	5	0	0.261	0.718	0.637***	8	4	0.526	0.724	0.273***	7	4	0.263	0.844	0.688***	9	0.350	0.768
He66	7	0	0.433	0.746	0.419***	5	0	0.407	0.698	0.416***	6	0	0.367	0.656	0.441***	7	0.402	0.706
Average	6.333	1.111	0.358	0.554	0.354***	6.000	1.444	0.373	0.567	0.341***	7.222	1.778	0.326	0.582	0.450***	9.333	0.352	0.569

Note: *A* = number of alleles; A_p = number of private alleles; H_o = observed heterozygosity; H_e = expected heterozygosity; *n* = number of individuals sampled; G_{IS} = inbreeding coefficient. *** $P<0.001$; ** $P<0.01$; *$P<0.05$; ns, not significant.

Supplemental Table S3. Single-locus and multi-locus pairwise and global *G*-statistics (G'_{ST}, Hedrick 2005) and corresponding p-values of 11 polymorphic microsatellites between three local populations of *Silene stellata*. *Note:* M = Meadow; R = Windrock; W = Woodland.

	M vs. R		M vs. W		R vs. W		Global	
Locus	G'st	P-value	G'st	P-value	G'st	P-value	G'st	P-value
1E	0.186	0.005	-0.020	0.968	0.161	0.141	0.128	0.042
S10	0.111	0.277	0.040	0.738	0.255	0.078	0.146	0.174
3R	0.016	0.191	-0.021	0.806	0.003	0.342	0.004	0.329
S12	0.096	0.129	-0.002	0.878	0.088	0.204	0.054	0.309
3S	0.412	0.006	0.197	0.135	0.434	0.045	0.340	0.008
S44	0.070	0.045	0.012	0.328	0.094	0.035	0.047	0.066
S23	0.098	0.008	0.030	0.115	0.075	0.019	0.064	0.005
S71	0.360	0.129	0.361	0.138	0.446	0.086	0.394	0.053
2G	0.214	0.136	0.284	0.012	0.652	0.005	0.411	0.001
3I	0.194	0.136	0.138	0.087	0.428	0.034	0.241	0.020
3J	0.156	0.225	0.267	0.102	0.229	0.350	0.206	0.190
Overall	0.146	0.002	0.081	0.089	0.215	0.002	0.148	0.001

Supplemental Table S4. Single-locus and multi-locus pairwise and overall *G*-statistics (G'_{ST}, Hedrick 2005) and corresponding p-values of 9 polymorphic microsatellites between three local populations of *Hadena ectypa*. *Note:* M = Meadow; R = Windrock; W = Woodland

	M vs. R		M vs. W		R vs. W		Global	
Locus	G'st	P-value	G'st	P-value	G'st	P-value	G'st	P-value
Hb02	-0.003	0.571	-0.026	0.950	-0.007	0.559	-0.007	0.559
Hb12	0.037	0.028	-0.015	0.820	0.044	0.079	0.044	0.079
Hb19	-0.010	0.939	0.017	0.293	-0.004	0.452	-0.004	0.452
Hb24	-0.003	0.460	-0.018	0.875	-0.005	0.515	-0.005	0.515
Hb29	-0.003	0.488	0.013	0.318	-0.016	0.791	-0.016	0.791
He58	0.010	0.107	0.010	0.185	0.012	0.185	0.012	0.185
He59	-0.001	0.508	-0.015	0.535	-0.006	0.613	-0.006	0.613
He62	-0.001	0.391	0.072	0.222	0.019	0.197	0.019	0.197
He66	-0.006	0.679	0.086	0.077	0.021	0.139	0.021	0.139
Overall	-0.001	0.502	0.008	0.250	0.005	0.255	0.005	0.255

Chapter 3: Sexually conflicting selection on floral design of the hermaphroditic plant *Silene stellata.*

Abstract

The evolution of traits may be governed by a balance of conflicting fitness advantages. Conflicting selection can be exerted by different selective agents as well as through differential response of various fitness components of the organism to the overall selective regime. In this study, we tested for conflicting selection between pollinator types and between male and female functions of the hermaphroditic plant, *Silene stellata*. The obligate moth *Hadena ectypa* that specializes on *Silene stellata* exerts both positive and negative effects on the plant by acting as both a pollinator and a seed parasite. The interaction is facultative in that multiple species of generalist moths also serve as equally effective pollinators for *S. stellata.* The strong fruit predation by *H. ectypa* larvae and the substantial pollination service of the generalist copollinator moths often makes the net outcome of this interaction negative, leading to the classification of this system as parasitic. Here, we quantified selection on floral and vegetative traits of *S. stellata* exerted predominantly by *H. ectypa* and the copollinators through female fitness, and through male fitness by assessing individual siring abilities using highly variable microsatellite markers. We found some support for differential selection exerted by *H. ectypa* vs. the copollinators. There was strong evidence of sexual conflict in the selection signal consistent across two years on petal length of *S. stellata*. Strong selection through

female function was detected to avoid fruit predation, while competition for mates through male function provided a counterbalancing force that may prevent the evolution of an “escape route” from a nocturnal pollination syndrome and contribute to the long-term maintenance of the *Silene-Hadena* interaction.

Introduction

Evolutionary biology has a long tradition of studying floral diversity as the consequence of adaptation to diverse animal pollinators. Beginning with Sprengel (1793) and Darwin (1862), similarity in floral form across unrelated lineages has been interpreted as evidence for convergent selection imposed by pollinators, a.k.a, pollination syndromes (Delpino 1867; Faegri and der Pijl 1966; Fenster et al. 2004; Rosas-Guerrero et al. 2014), shedding light on the importance of pollinator interactions in floral evolution.

A major advance in our understanding of floral evolution is the recognition that phenotypic selection on floral forms is a complex and multi-faceted process. Instead of the simplistic picture of pairwise plant-pollinator interactions, empirical studies have revealed the prevalence of diffuse selection, where selection on floral traits is determined by a community of taxa that interact with the focal plant species (Hougen-Eitzman and Rausher 1994; Iwao and Rausher 1997; Inouye and Stinchcombe 2001; Strauss and Irwin 2004; Strauss et al. 2005). Beyond the recognition of the complexity of pollinator communities potentially acting as selective agents for floral evolution, research has also demonstrated an important role of antagonist-mediated selection in comparison with pollinator-mediated selection on floral traits. Those studies encompass a variety of interactions including herbivory, nectar robbing, predispersal seed predation, and host-pathogen effects (reviewed by Strauss and Whittall 2006). When antagonistic and mutualistic interactions both impose direct or indirect selection on floral traits, conflicting selection can emerge as the result of divergent phenotypic optima favored by the positive

vs. negative interactions, leading to evolutionary trade-offs. Furthermore, direct or indirect interactions between selective agents can lead to non-additive selection deviating from the superposition of selection pressures exerted by individual taxa in the absence of others (Strauss et al. 2005). For example, herbivory has been shown to have negative effects on pollinator visitation in some cases (Lehtilä and Strauss 1997; Mothershead and Marquis 2000; Hamback 2001), but exhibit positive effects in other cases (Poveda et al. 2003). An extreme scenario of the predominance of antagonistic selection occurs when pollinator selection is completely negated by the swamping effect of herbivory (Gómez 2005).

Another largely unexplored factor of floral trait evolution may be selection through male reproductive success. Approximately 90% of flowering plants exhibit hermaphroditic sex expression (Barrett and Hough 2013), where total individual fitness is the sum of two fitness components realized separately through male and female functions. However, most natural selection studies of hermaphroditic plants have quantified selection using various female fitness components as the proxy for total fitness (a search on BIOABS database of Web of Science for the topics “natural selection” and “floral traits” returns a total of 51 records, of which only 7 assessed male reproductive success). Inference of evolutionary consequences based on female reproductive success alone is only appropriate when the phenotypic optima for the two sexual functions overlap or when selection through male function is relatively weak. However, evolutionary theory has suggested the possibility of prevalent conflicting selection on floral traits across sexual functions for hermaphroditic plants (Morgan 1992). Moreover, some have suggested competition for male mating success as the dominant force

underlying floral evolution (Bateman 1948; Arnold 1994). The majority of the small number of studies examining selection on floral design for both sexual functions showed a lack of conflict (Conner et al. 1996; Morgan and Schoen 1997; Delph and Ashman 2006; Hodgins and Barrett 2008; Sahli and Conner 2011; La Rosa and Conner 2017). However, fewer studies have quantified male reproductive success by directly examining siring ability using genetic markers (e.g., Conner et al. 1996, Hodgins and Barrett 2008, Sahli and Conner 2011). Instead, most studies have used pollen removal as a surrogate measure for male reproductive success. While the correlation between siring success and pollen export has been confirmed in a few systems (Galen 1992; Nilsson et al. 1992; Ashman 1998), it has been rejected in a number of other studies (e.g., Kobayashi et al. 1999, Conner et al. 2003). Consequently, we have a very limited understanding of whether selection via male fitness is a dominant force influencing floral evolution.

A complex picture emerges if one considers both the sexual dichotomy and the multitude of biotic or abiotic factors potentially serving as selective agents for hermaphroditic plants. Differential sensitivity of male vs. female function to different selective agents could result to sexually conflicting selection, as suggested by several studies (Stanton and Preston 1988; Galen and Stanton 1989; Campbell et al. 1991). For instance, a floral trait positively correlated with both pollinator visitation and seed predation can be under primarily negative selection through female function if pollen limitation is minimal. However, for outcrossing plants, a large portion of male reproductive success is realized on other plants following pollen removal and therefore is less likely to be swamped by the antagonistic effect. Therefore, even with evidence supporting a more important role of antagonistic selective agents for floral evolution with

respect to female reproductive success, the impact of pollinator-mediated selection could be maintained through plant competition for male reproductive success.

In this study, we examine and compare selection on floral traits mediated by parasitic and mutualistic interactions, and through male and female function, using a North American *Silene-Hadena* system. Facultative interactions are commonly found between nocturnal moths from the genus *Hadena* and plants of the Caryophyllaceae family, especially in the genus *Silene* (Brantjes 1976*a*, 1976*b*; Kephart et al. 2006; Giménez-Benavides et al. 2007; Reynolds et al. 2012; Prieto-Benítez et al. 2016). The nocturnal *Hadena* moths act as the pollinating seed predator for various *Silene* species, along with the generalist copollinating moths. The interaction can range from mutualistic to parasitic depending on the net benefit from the *Hadena* moths (Dufaÿ and Anstett 2003; Kephart et al. 2006). The low degree of specialization of the *Silene-Hadena* interaction has made it an emerging model system for our understanding of the role of ecological context in the evolution of mutualisms and coevolution between pollinators and plants (Kephart et al. 2006; Bernasconi et al. 2009). Additionally, various sexual systems are found in the *Silene* species that interact with *Hadena*, including hermaphroditism, dioecy and gynodioecy (Casimiro-Soriguer et al. 2015). It is therefore intriguing to explore whether *Hadena* is a driving factor for sexual system evolution in *Silene.*

Specifically, we quantified selection on floral traits of *S. stellata* exerted by *H. ectypa* and the copollinators by taking advantage of the temporal separation of activity peaks of these two groups. For each pollinator group, in addition to female fitness, we measured selection through male fitness by directly assessing siring success using highly

polymorphic microsatellite markers. We focus on three questions: (1) Do *H. ectypa* and the copollinating moths exert divergent floral selection on *S. stellata*? (2) Does the dual role of *H. ectypa as* pollinator and seed predator cause sexually conflicting selection on the hermaphroditic flowers of *S. stellata*? (3) What role does the potentially conflicting selection play on the coevolution of the *Silene-Hadena* interaction?

Methods

Study system

The study system includes the North American hermaphroditic plant *Silene stellata*, its obligate pollinating seed predator, the noctuid moth, *Hadena ectypa*, and a group of copollinating nocturnal moths, including the noctuid moths *Amphipoeaea americana Feltia herelis*, *Autographa precationis* ,and *Cucullia asteroids*, the arctiid *Halysidota tessellaris*, and the notodontid, *Lochmaeus manteo*(Reynolds et al. 2009). *Silene stellata* L. is an infrequent, iteroparous, long-lived perennial herb that is distributed throughout the eastern United States. Flowering of *S. stellata* occurs from early July through early September and is characterized by panicle inflorescences with white, hermaphroditic, protandrous flowers (average of 25 ovules/pistil, Reynolds et al. 2009). An individual plant at our study site usually produces multiple stems and on average produces ~40 flowers each flowering season (Reynolds et al. 2012). Outcrossing rate is relatively high (>73%, Reynolds 2008).

Hadena ectypa is the obligate pollinating seed predator of *S. stellata*. It is distributed from Massachusetts west to Minnesota and Kansas, and south to northern

Georgia, concordant with the distribution of *S. stellata* (Schweitzer et al. 2011; Nelson 2012). Adult male and female *H. ectypa* extract nectar in the flowers of *S. stellata* with pollination taking place simultaneously. Oviposition behavior follows nectaring, as female moths lay single or multiple eggs at the base of the ovary or on the ovary wall (Zhou et al. 2016*a*). Forty percent of all visits are followed by oviposition (Kula et al. 2013). Newly hatched larvae bore into the ovary and develop through the third instar therein, before starting to forage between flowers and plants. A larva can consume up to 40 flowers and/or unhardened fruits under lab conditions (Reynolds et al. 2012). The larvae pupate underground, and *H. ectypa* exhibits a univoltine life history at our study sites. Previous work has shown female *H. ectypa* preference for long corolla tube length in *S. stellata*, as well as a significant negative correlation between corolla tube length and seed set of *S. stellata* (Kula et al. 2013), indicating potential negative selection on floral traits through female function. The copollinating generalist moths are equally effective in pollen transfer (Reynolds et al. 2012) but do not exhibit seed predation, that is, they are strict mutualists.

Field experiment

To quantify selection on floral traits of *S. stellata*, through both male and female reproductive success (RS), field experiments were conducted in 2012 and 2013 in an open meadow under a power line cut near the University of Virginia's Mountain Lake Biological Station in Giles County, Virginia, U.S.A. (37.3471, –80.5426, elevation ≈ 1,100–1,300 meters). The experimental plot was near the top of a naturally occurring population around which a 20 m × 20 m fenced exclosure was built to prevent deer herbivory. Adult *H. ectypa* abundance peaks early in the flowering season, when

copollinator species are rare (Reynolds et al. 2012), and drops quickly as copollinating moths become the primary pollinators. Therefore, in each year, two experiments were conducted during *Hadena*-dominant and copollinator-dominant periods in order to quantify selection pressures exerted predominantly by the *H. ectypa* and the copollinators moths, respectively. Each experiment was carried out for a week with an experimental population consisting of ~60 equally spaced adult plants. The experimental plot was set up two weeks prior to the onset of the "early" experiment. In order to prevent pollen flow from outside of the study population, a 10 meter exclusion zone around the exclosure was created by removing all flowering stems of *S. stellata* plants in this area. Since most *S. stellata* pollen carryover distances are only 1-2 meters (Reynolds et al. 2009, and confirmed in Chapter 1), the exclusion zone was expected to prevent pollination by unsampled plants on the outside of the exclosure.

We divided the plot up into eight transects along the elevation gradient. Each transect was further divided into eight evenly spaced quadrants containing multiple *S. stellata* plants with a radius of 1 m. All plants outside the quadrants were removed. For each experiment, we randomly picked one *S. stellata* plant from each quadrant and removed all non-experimental plants that were flowering or carried flower buds expected to open within one week. We kept a number of non-flowering experimental plants in each quadrant to be used as backups or used in the second experiment. One week prior to the onset of the "late" experiment, a second experimental population of the same size was set up by randomly selecting, from each quadrant a new flowering plant or one bearing large flower buds on course to open within one week. To avoid repeated measurements, plants that were used in a previous experiment or in the previous year were excluded from the

new experiment. A permanent metal label was installed at the base of each study plant for future identification.

We confirmed the dominant status of *H. ectypa* and the copollinator moths during this study by conducting pollinator surveys throughout the flowering season. Briefly, following Reynolds et al. (2012), we conducted walks every night when weather conditions favored moth activity such that 20 patches of *S. stellata* plants were randomly chosen along predefined transects. In each patch we counted the number of *H. ectypa* and copollinating moths contacting *S. stellata* flowers during a one-minute period. Number of open flowers per patch was estimated as the product of the total number of plants in the patch and the number of open flowers on a randomly chosen plant. We calculated moth density in a given day as the ratio between total number of *H. ectypa* or copollinators observed and the total number of flowers summed over all patches. We also assessed the density of adult *H. ectypa* by quantifying oviposition activity of *H. ectypa* that has been shown to be a good indicator of adult *H. ectypa* activity (Reynolds 2008). For each day, we dissected ~20 *S. stellata* flowers collected from the same site and counted *H. ectypa* eggs. The average number of eggs per flower was used to represent *H. ectypa* oviposition activity of the night before the collection date (Figure 1, 2).

All flowers initiated during the one-week experimental period were tagged in their male phase during the first day of opening. We followed each labeled flower for three consecutive days (one day in male phase and two days in female phase). For each day, we counted the number of eggs in the flower using a jeweler's eyepiece. Oviposition became rare in the late experiment, therefore, we only carried out egg counting during the early experiment for each year.

Traits studied

Phenotypic measurements were carried out on the experimental plants with a caliper (0.1 mm) for seven floral traits: (1) length of the corolla tube (TL); (2) width of the corolla tube (TW); (3) length of the largest petal (PL); (4) width of the largest petal (PW); (5) number of fringes on the distal margin of a randomly chosen petal (FR); (6) distance from the nectary at the base of the flower to the tip of the anther (nectar-anther distance); (7) nectar-stigma distance. Anther exsertion (AN) or stigma exsertion (ST) was calculated as the difference between nectar-anther or nectar-stigma distance and corolla tube length. All floral traits except ST were measured on flowers during the first day of opening (the male phase). Stigma measurements were taken on the first day of the female phase. On average, five of the labeled flowers were measured on each plant. Repeatability (Falconer and Mackay 1996) of the seven floral traits were all significantly different from zero and ranged between 0.39 (ST) to 0.89 (PL) (see Chapter 4). Additionally, we recorded the total number of flowers open throughout the experimental period (NF) and height of the longest stem (HT). Means of floral-trait measurements from all measured flowers on a plant were calculated to obtain one representative measure for each trait per plant per year. The mean trait measures were z-transformed within each experiment to mean = 0 and variance = 1.

Female fitness components

We harvested all labeled reproductive units (RUs) from the study plants after fruit maturation but before fruit dehiscence. In the laboratory, all pistils were scored for whether they initiated a fruit, had been predated by *H. ectypa* larvae, or matured a fruit without experiencing predation. The proportion of flowers to initially form fruits

(hereafter referred to as "fruit initiation rate") is indicative of the portion of flowers that were pollinated (there is little fruit abortion in *S. stellata*) and was calculated as the total number of initiated fruits divided by the total number of labeled flowers. For RUs not consumed by *H. ectypa* larvae, we determined that fruit initiation had occurred if the ovary was enlarged while a shriveled ovary was indicative of its absence. Larvae sometimes consume the entire pistil of flowers and leave only the petals and calyx (flower predation). In such cases, it was impossible to determine if the RU was enlarged or not. Therefore we excluded such RUs from the calculation of fruit initiation rate. If an initiated fruit was consumed late in its development by *H. ectypa* larvae, the hardened ovary wall was left in place with a feeding hole that was 1 to 2 mm in diameter. We used the number of successful fruits (fruit set) as the measure of female RS, where a successful fruit must have been initiated and free from *H. ectypa* predation. Fruit set has been shown to be highly correlated with seed set in *S. stellata* (Pearson correlation coefficient r=0.88, p<0.001; Reynolds et al. unpublished data) thus is an adequate measure of female RS.

Paternity analysis

Leaf tissue was collected from each of a total of 227 adult *S. stellata* experimental plants and stored in silica gel. In order to obtain sufficient amounts of genomic DNA from progeny for subsequent amplifications, seeds from all successful fruits were first sowed and cold stratified during the winters of 2012 and 2013, and then germinated in a greenhouse environment in the following springs, respectively. Seedlings of 2012 were harvested into silica gel for subsequent DNA extraction, while DNA of seedlings of 2013 were preserved on Whatman FTA® cards (Whatman Inc., USA). Due to seed predation by *H. ectypa* larvae, only 42 percent of the tagged flowers yielded mature fruits with at

least one seed. A total number of 2371 seedlings were successfully germinated and preserved (mean number of seedlings collected per plant = 10.4).

Adult plant and the 2012 seedling DNAs were extracted using the Autogen Plant Kit, following the manufacturer's protocol. DNAs were extracted from the 2013 seedlings using the FTA® purification reagent following a modified extraction protocol (Siegel et al. 2017). Plants were genotyped for 11 novel microsatellite loci designed for *S. stellata* (Zhou et al. 2016*b*). DNA amplification was performed with fluorescently labeled forward primers (FAM- or HEX-) in 10-µL reactions, using the QIAGEN Type-it® Microsatellite PCR Kit. Each reaction contained the following components: ~10ng of genomic DNA, 5 µL of the 2x Multiplex PCR Master Mix, 1µL of the forward primer, and 1µL of the reverse primer (final concentration of the primers: 0.2µM). A touchdown PCR protocol was performed in a BIO-RAD T100 thermocycler (Bio-Rad, Hemel Hempstead, UK) using the following conditions: 95°C for 5 min; 5 cycles at 95°C for 30 s, 60°C for 1.5 min, and 72°C for 30 s; 28 cycles at 95°C for 30 s, 55°C for 1.5 min, and 72°C for 30 s; and a final extension at 60°C for 30 min. PCR products were diluted in nuclease-free water (dilutions ranged from 1:10 to 1:50), and 1 µL of each dilution was added to 9 µL of HiDi formamide with 1 µL ROX standard (DeWoody et al. 2004). Samples were heated to 95 °C for six minutes, cooled to 4 °C for six minutes, and loaded onto an ABI 3730xl automated capillary sequencer with a 50 cm, 96 channel array containing POP-7 polymer for fragment analysis at the Laboratories of Analytical Biology (LAB) of the Smithsonian National Museum of Natural History. Fragment patterns were then visualized and scored using the GeneMapper V3.7 software (Applied Biosystems, Foster City, CA, USA). Continuous product sizes were exported and

rounded to multiples of repeat number (binned) using the software Tandem (Matschiner and Salzburger 2009).

We assigned paternity of individual seedlings categorically to the candidate sires using the paternity exclusion program PolyPatEx (Zwart et al. 2016). The set of candidate sires consisted of all adult study plants of a given experimental period. Given that *S. stellata* is a tetraploid, the underlying genotypes of partial heterozygotes cannot be readily resolved (for example, an individual showing two alleles A, B on a given locus can have AAAB, AABB or ABBB as the underlying genotype). PolyPatEx is designed for polyploids and resolves this challenge by assessing the genotypic compatibility between the progeny, the mother and the candidate sires through the enumeration of all possible allelic configurations given their allelic phenotypes (presence or absence of alleles). Paternity analysis using "PolyPatEx" was carried out in the R environment (R Core Team 2016). We used the number of seedlings sired as the measure of male fitness in subsequent statistical analyses.

Testing for selection differences between pollinator types and female and male function

We used analysis of covariance (ANCOVA) to test for differences in selection gradients between pollinators and male and female functions (sex). The full ANCOVA model contains nine trait main effects as well as the main effects of sex and pollinator type, trait × sex and pollinator × sex interactions and the three-way sex × pollinator × trait interactions. This model contains 40 parameters and the sample size for each year was relatively small. Thus, to avoid over parameterization and to test if certain interactions were important predictors, we followed a model selection procedure using the Akaike Information Criteria (AIC). Specifically, in addition to the full model, we also fit and

compared the performance of four models where the nonzero parameters are (1) main effects, (2) main effects + trait × pollinator interactions, (3) main effects + trait × sex interactions, (4) main effects + trait × pollinator interactions + trait × sex interactions. Male and female fitness scores were scaled to mean fitness of a particular pollinator-by-sex combination such that the main effects of sex and pollinator and sex × pollinator interaction were eliminated from the model and only the standardized selection gradients were compared across treatment levels.

The same sets of models were run for each year separately. We interpreted significant trait × pollinator interaction as an indication that *H. ectypa* and the copollinator moths exert different selection. Significant trait × sex interaction indicated that selection differed between the two sexual functions. All ANCOVA analyses were performed in R using the function "lmer" with "plant" included as a random effect.

Estimating selection gradients

We performed separate multiple regressions to quantify selection gradients for each of the eight pollinator × sex × year combinations. Due to the relatively small sample size of each experiment, we only focused on quantifying the linear selection gradients. For the two early experiments during peak adult *H. ectypa* activity with egg count data, we also modeled mean number of eggs per flower as a linear function of the above predictor variables to quantify oviposition preference of female *H. ectypa*. Multiple regression analyses were carried out in R using the function glm. Fitness scores were scaled to the mean for each model in order to generate standardized selection gradients. At the same time, since the untransformed fitness scores were count data, we also fit the GLM by specifying quasi-Poisson distribution for the residuals with the log link function. Results

of both the Gaussian and quasi-Poisson models were reported. We chose the quasi-Poisson over the ordinary Poisson model as the residual distribution because there was evidence of overdispersion of residuals around the predicated mean.

Since *S. stellata* is partially outcrossing, we quantify and present selection gradients through male function using both the total number of seedlings sired including those through selfing (total fertility) and those sired exclusively through outcrossing (outcrossing fertility). Given the prevalence of inbreeding depression in plants and the fact that *S. stellata* is a predominantly outcrossing species, we used selection quantified through outcrossing male fertility to test for conflicting selection on traits between sexual functions. To test for conflicting selection between sexual functions averaging over the two pollinator types, we also conducted multiple regressions using data pooled across the "early" and "late" experiments for a given year. Male and female fitness scores were scaled by the mean of a given sex for each experiment, therefore eliminating the pollinator main effect. We ran two separate Gaussian models as above, with relative fitness as the response variable to generate standardized selection gradients.

Results

Due to plant mortality, on average our experiments consisted of ~57 replicate plants (2012 early: N = 59; 2012 late: N = 58; 2013 early: N = 55; 2014 late: N = 55; see Table 1). We measured 1094 flowers across two years (total number of flowers measured, mean number measured per plant: 2012 early: N = 280, 4.8; 2012 late: N = 239, 4.1; 2013

early: N = 294, 5.4; 2014 late: N = 281, 5.1). The difference in flower numbers across years was most likely caused by the difference in weather condition.

Observed phenotypic correlations ranged between 0.00 and 0.70. The strongest correlation was between PW and PL (Table 2). The two traits HT and NF showed significant positive correlation with each other in all experiments. Correlation between floral and the two vegetative traits was generally low (Table 2). Fruit initiation rate was high (mean ± SE = 0.92 ± 0.15, Table 1) and did not significantly differ among the four experiments ($F = 0.47$, $df = 1$, $P > 0.10$) nor between years when data were pooled across pollinator types ($F = 0.22$, $df = 1$, $P > 0.10$). An ANOVA test showed significant differences in fruit predation rates among experiments ($F = 48.1$, $df = 1$, $P < 0.001$) and was the highest in early 2012 (mean ± SE = 0.59 ± 0.36, Table 1) and lowest in late 2013 (0.19 ± 0.24). When averaging across pollinator types, the predation rate was significantly higher in 2012 ($F = 45.35$, $P < 0.001$) than 2013.

We genotyped a total of 2371 seedlings with a mean of 593 seedlings per experiment (2012 early N = 583; 2012 late N = 773; 2013 early N = 545; 2013 late N = 470. See Table 1), yielding an average of 10.4 seedlings genotyped per plant. The mean percentage of seedlings with unambiguous paternity was 92%, resulting in 2181 seedlings assigned categorically to unique fathers.

Conflicting selection between pollinator types and female and male function

We quantified selection through male function using number of seedlings sired including those from selfing (total fertility) and those sired exclusively through outcrossing (outcrossing fertility). Selection gradients were generally similar and significantly correlated for three of the four experiments, except late 2012. Multiple regressions based

on outcrossing fertility produced more significant terms than total fertility (Table 3, 4). Gaussian and quasi-Poisson models generally produced similar P-values (Table 3, 4). We present significance levels of terms based on Gaussian and quasi-Poisson models along with the standardized selection gradients (Table 3, 4). Given that our ANCOVA models were fitted with Gaussian-distributed residuals and that the Gaussian GLM models produced more conservative P-values (Table 3, 4), comparisons of selection gradients across pollinator types and sexual functions were based on P-values of the Gaussian multiple regressions unless otherwise noted.

The AIC model comparison strongly supported model 3 (main effects + trait × sex interactions) across years: difference in the AIC scores between model 3 and the second best model was > 10 in both years (Table 5). Therefore we only present the ANCOVA results and interpret differential selection gradients (β) based on model 3.

In both years, number of flowers (NF) was a significant main effect (Table 6). In 2012, significant difference in selection between sexual functions was detected for three traits: petal width (PW), petal length (PL), number of petal fringes (FR), while NF × sex interaction only trended towards significance (P = 0.096; Table 4).

In the early experiment of 2012 when *H. ectypa* was dominant, there was significant negative selection for PW ($\beta = -0.77$, P = 0.016; Table 3) through male function. Selection for PW reversed sign and was marginally significant through female function (PW: $\beta = 0.30$, P = 0.088). We found significant positive selection for PL through male function ($\beta = 0.72$, P = 0.034), while selection through female function was significantly negative ($\beta = -0.40$, P = 0.037).

Selection gradients for PW and PL became insignificant for the late experiment when the copollinators were dominant. When the 2012 data were pooled across pollinator types, we detected significant selection on the petal dimensions through male function (PW: $\beta = -0.40$, P = 0.035; PL: $\beta = 0.44$, P = 0.022, Table 7). The selection gradient through female function based on the pooled dataset was positive but insignificant on PW ($\beta = 0.17$, P = 0.18), while negative selection on PL was negative and trended toward significance ($\beta = -0.22$, P = 0.089).

Selection for FR was not significant for any pollinator-by-sex combinations in 2012 according to the Gaussian model. However, there was significant selection on FR through male function in the early experiment according to the quasi-Poisson model ($\beta = 0.36$, P = 0.012; Table 3). When data were pooled across pollinator types, selection on FR was of opposite signs through the two sexual functions but not significant (male: $\beta = 0.22$, P = 0.134; female: $\beta = -0.14$, P = 0.157; Table 7).

Selection for NF was positive and significant for all four pollinator-by-sex combinations in 2012, and when averaged across pollinator types, selection on NF through female function was stronger than through male function (NF) (male: $\beta = 0.35$, P = 0.012; female: $\beta = 0.62$, P < 0.001; Table 7).

In 2013, we observed significant trait × sex interactions for PL and NF (Table 6). In general, selection on the two petal dimensions was of opposite signs through male vs. female functions, similar to the results of 2012.

In the early experiment of 2013, there was significant positive selection for PW through female function ($\beta = 0.24$, P = 0.047; Table 6), while selection for PW through male fitness was negative but insignificant ($\beta = -0.183$, P = 0.192).

Selection on PL was negative and marginally significant (β = –0.24, P = 0.063) through female function in the early experiment. Significant positive selection was detected through male function (β = 0.363, P = 0.018) in the same period.

When data were pooled across pollinator types, there was marginally significant selection of opposite signs on PL through both functions (male: β = 0.30, P = 0.051; female: β = –0.13, P = 0.099; Table 7). Selection on PW through female fitness was positive and trended toward significance using the pooled data (β = 0.14, P = 0.074).

We found significant positive selection for FR in the early experiment through male fitness (β = 0.26, P = 0.014; Table 4), similar to the results of 2012. However, we did not find significant FR × sex interaction.

Selection on NF was not significant through male function in 2013 (total selection over pollinator types: β = 0.084, P = 0.426; Table 7), while higher NF was favored through female fitness in both the early and late experiments (total selection over pollinator types: β = 0.55, P < 0.001).

Additionally, there was a significant negative selection for corolla tube width (TW) through male function in the early experiment (β = –0.27, P = 0.010). Averaging over pollinator types, selection on TW through male function was positive but insignificant (β = –0.18, P = 0.164. Table 7), while selection through female function was close to zero (β = 0.05, P = 0.501).

When pooled across years and genders, absolute values of β ($\overline{|\beta|}$) were significantly higher in the early experiments than the late experiments (early: $\overline{|\beta|}$= 0.23, late: $\overline{|\beta|}$= 0.13, Wilcoxon rank sum W = 823, P = 0.049). When selection gradients were pooled across years and pollinator types, selection through male function was

significantly stronger than through female function (male: $\overline{|\beta|}$= 0.21, female: $\overline{|\beta|}$= 0.15, W = 823, P = 0.046).

Oviposition preference of Hadena ectypa

A Welch two sample t-test showed the mean number of eggs per flower was significantly higher in 2012 than 2013 (2012: Mean ± SE = 1.04 ± 0.82; 2013: Mean ± SE = 0.55 ± 0.32; t = 4.27, P < 0.001). A Spearman rank test showed significant negative correlation between oviposition and female fitness across years (2012: ρ = –0.26, P = 0.049; 2013: ρ = –0.40, P = 0.003). However, correlations between the mean number of eggs per flower and male fitness was close to zero and insignificant in both years. Multiple Gaussian regression of plant trait on mean number of eggs per flower showed the only significant predictor of oviposition rate to be NF in both years (Table 8).

Discussion

Although sexual conflict has received a tremendous amount of attention in animal systems, evidence of its presence in plants has rarely been explored (a search on BIOABS database of Web of Science for topic "sexual conflict" returns a total of 887 records when restricted to animals, but only 11 when restricted to plants).

We find support for sexually conflicting selection on the floral traits of *Silene stellata*. The strongest evidence comes from petal length (PL), for which there is consistent significant positive selection through male function across years, and consistent significant negative selection through female function. The signal is strongest

in the early experiment for both years, when *Hadena ectypa* is the dominant pollinator for *S. stellata*. Selection on PL is insignificant in the late experiment when the primary pollinator transitioned to the copollinating moths that are purely mutualistic. However, when data are pooled across experiments within a year, we are still able to detect significant or marginally significant selection gradients of opposite signs on PL (Table 7).

There is some evidence of conflicting selection on petal width (PW), for which we detect significant positive selection through female function across years and negative selection through male function significant in one year. There are also other significant trait × sex interactions including number of fringes (FR) and number of flowers (NF). However, for these traits, selection gradients are either only significant in one sex or only differ in strengths instead of signs, and therefore do not constitute evidence for conflicting selection.

Male fitness is only correlated with floral traits when selfed seeds are not included in the analysis. In chapter 1 I demonstrated high outcrossing and significant inbreeding depression in *S. stellata*, consistent with the related species *Silene virginica* (Dudash and Fenster 2001). Therefore, we believe selection quantified through outcrossing male fertility is more likely to reflect actual selection pressure in the natural environment and more suitable to predict the response to selection.

We find little evidence of divergent selection pressures exerted by the two pollinator types based on the AIC scores of the ANCOVA model 2 (Table 5). Selection in the early experiment is more frequently significant and stronger in magnitude than the late experiment. The lack of trait × pollinator interaction could be due to the incomplete separation of the two cohorts of pollinators. Therefore, for each experimental period, we

recorded selection exerted by a mixture of *H. ectypa* and the copollinators (Figure 1, 2). In this case, the shift of *H. ectypa* density would have caused the observed difference in the significance and strength of selection. Alternatively, when we average selection over the sexual functions, the total selection gradient tends toward zero because of the conflicting selection pressures observed. This could compromise the ability of a model without sex as a predictor variable to detect selection that differs between pollinator types through the detailed response of each gender. For example, if selection on a trait in the early experiment was positive through male function and negative through female function, while in the late experiment it reversed signs, signals of conflicting selection across pollinator types could be very weak averaging across sexual functions, although selection is actually of opposing signs through each function.

Owing to the concern of over fitting the model given the relatively small sample size, we do not present the result of the full model incorporating the three way sex-by-pollinator-by-trait interactions for a given year. However, when data are pooled across years to increase statistical power, we do observe significant three-way interactions in four traits (Supplemental Table 1), in addition to the significant sex-by-trait interactions observed using model 3, providing some evidence for differential selection by *H. ectypa* vs. the copollinators. However, we find no significant selection through male or female function in the late experiment on traits except NF. This suggests the significant three-way interactions most likely result from differences in the strengths of selection between pollinator types, instead of signs.

The conflict of trait function between sexes discovered here is contrary to most previous studies of plant-pollinator interactions (e.g., Delph and Ashman 2006; Sahli and

Conner 2011; La Rosa and Conner 2017). However, our study system includes the negative interaction of seed predation by the *H. ectypa* larvae in addition to the positive pollinator interactions. Given the consistently high fruit initiation rate and the lack of evidence for pollen limitation in *S. stellata* (unpublished data), selection through female fitness is likely to be weak through differential pollinator visitation. Instead, the high fruit predation rate as well as its high variation among plants suggests selection through female fitness could be mainly exerted through predation by the *H. ectypa* larvae. This is in line with the findings by Burkhardt *et al.* (2012) where for the dioecious *Silene latifolia*, its pollinating seed predator *Hadena bicuris* exerts more selective force on female plants through predation than pollination. We find significant negative correlations between oviposition rate and fruit across years. However, with the exception of NF, there are no floral traits associated with higher oviposition rate, in contrast to a previous study where numerous floral traits were found to be associated with oviposition rate (Kula et al. 2013). The lack of correspondence between floral traits and oviposition rate detected in our experiments suggests that in the years of our study *S. stellata* floral traits are mainly selected through female function for the avoidance of secondary predation by *H. ectypa* larvae, instead of oviposition itself.

The high fruit initiation rate and its low variation makes it unsuitable as a measure of pollinator visitation rate for this study. The selective advantage of longer and narrower petals through male fitness could be either for pollinator attraction or for pollen transfer efficiency. The significant selection for more fringes at the distal end of the petals seems to indicate a trait profile that helps to increase the contrast between the flower and the background under low light conditions, whereas the significant selection for narrower

corolla tube implies petal shape could be involved in ensuring higher precision or efficiency in pollen export (Campbell et al. 1991; Fenster et al. 2009). Detailed pollinator visitation data will help to elucidate the exact mechanism of selection through male function in the future.

Conflicting selection from non-pollinator agents has been documented in a number of systems (reviewed by Strauss and Whittall 2006). In our system the same species serves as both a pollinator and a non-pollinator selective agent (predator) in its different life stages. Furthermore, male and female fitness components of the plant potentially have differential sensitivity to these two types of interactions, giving rise to the observed conflict between male and female function. The observed sexually conflicting selection has profound implications for the evolutionary dynamics of the *Silene-Hadena* systems. The role of *H. ectypa* was previously classified as parasitic due to the large quantities of fruits a larva consumes as well as to the presence of mutualistic copollinators that are equally effective at pollen transfer (Reynolds et al. 2012). Consequently, an "escape route" from *H. ectypa* or nocturnal pollination syndrome in general should be evolutionarily favored (Kephart et al. 2006). Here, we show that the same floral traits potentially under selection to avoid fruit predation could be under opposing selective pressure to increase male fertility. Reynolds *et al.* (2012) showed *H. ectypa* to be a substantial pollinator of *S. stellata* along with the copollinating moths. Therefore, selection to increase male siring ability through interaction with *H. ectypa* could provide a strong counterbalancing force to keep the *S. stellata* population at an evolutionary equilibrium and contribute to the long-term maintenance of this interaction. Furthermore, the genus *Silene* exhibits diverse sexual systems including

hermaphroditism, gynodioecy, and dioecy (Casimiro-Soriguer et al. 2015). Phylogenetic analyses demonstrate that there have been at least two independent evolutionary occurrences of dioecy in the genus (Desfeux et al. 1996). The sexually conflicting selection pressures in the hermaphroditic *S. stellata* could therefore be a mechanism promoting individual specialization of certain sexual functions or even the transition from hermaphroditism to gynodioecy or dioecy.

Table 1. Summary statistics of male and female fitness components of *Silene stellata* near Mountain Lake Biological Station assessed during the early (*Hadena ectypa* dominant) and late (copollinators dominant) flowering season for 2012 and 2013. SE = standard error.

Experiment	Adult plants	Seedlings genotyped	Male fertility (Mean ± SE)	Fruit initiation rate (Mean ± SE)	Successful fruits (Mean ± SE)	Predation rate (Mean ± SE)
2012 Early	59	583	9.57 ± 11.52	0.91 ± 0.19	2.66 ± 2.95	0.59 ± 0.36
2012 Late	58	773	12.47 ± 11.11	0.92 ± 0.19	3.91 ± 4.13	0.41 ± 0.35
2013 Early	55	545	9.28 ± 7.11	0.93 ± 0.1	9.77 ± 6.91	0.2 ± 0.3
2013 Late	55	470	10.29 ± 9.84	0.93 ± 0.1	8.6 ± 7.01	0.19 ± 0.24
Overall	227	2371	10.24 ± 11.52	0.92 ± 0.15	6.36 ± 6.34	0.34 ± 0.35

Table 2. Phenotypic Pearson-correlation matrices of the nine traits measured in the early (upper diagonal) and late flowering season (lower diagonal) of *Silene stellata* near Mountain Lake Biological Station in 2012 and 2013.

	2012								
	TL	TW	PW	PL	FR	ST	AN	HT	NF
TL		0	0.3*	0.22	0.16	-0.06	0.09	-0.06	0.21
TW	-0.19		0.42***	0.54***	0.29*	0.18	0.57***	0.05	0
PW	0.27*	0.44***		0.59***	0.45***	-0.06	0.28*	0	0.09
PL	0.28*	0.51***	0.61***		0.08	0.15	0.33**	-0.07	-0.01
FR	0.02	0.02	0.26*	-0.03		0.15	0.35**	0.03	-0.03
ST	-0.09	0.35**	0.17	0.19	0.28*		0.44***	0.14	0.16
AN	-0.05	0.54***	0.37***	0.51***	0.09	0.38***		0.21	0.04
HT	0.07	0.1	0.27*	0.11	0.17	0.03	0.21		0.36**
NF	0.01	0.02	0.12	0.2	0	-0.06	0.22	0.47***	
	2013								
	TL	TW	PW	PL	FR	ST	AN	HT	NF
TL		0.08	0.33**	0.4***	0.04	-0.03	-0.15	0.07	-0.13
TW	-0.09		0.46***	0.42***	0.09	0.21	0.31*	-0.18	-0.07
PW	0.09	0.31*		0.7***	0.16	0.01	0.04	-0.08	-0.09
PL	0.16	0.38***	0.69***		0.07	0.02	-0.06	-0.03	-0.11
FR	0.03	0.31*	0.4***	0.18		0.08	0.05	-0.05	-0.07
ST	-0.28*	0.26*	0.34**	0.47***	0.17		0.29*	-0.03	0.06
AN	-0.01	0.31*	0.32*	0.42***	0.21	0.62***		0.11	0.08
HT	0.04	-0.04	0.07	0	0.06	0.08	0.25		0.42***
NF	-0.25	0	0.16	-0.02	0.02	0.22	0.24	0.37**	

PL: petal length; PW: petal width; FR: number of petal fringes; TL: corolla tube length; TW: corolla tube width; ST: stigma exsertion; AN: anther exsertion; HT: display height; NF: number of flowers.

*** $P<0.001$; ** $P<0.01$; *$P<0.05$

Table 3. Results of generalized linear multiple regressions through male and female functions during early and late flowering season of 2012 for *Silene stellata* near Mountain Lake Biological Station. P-values based on both Gaussian (Pg) and quasi-Poisson (Pq) models are shown next to the standardized linear regression coefficients (β). Gaussian models were performed using relative fitness as the response, while quasi-Poisson models were based on untransformed count data. Selection through male function was quantified using both outcrossing fertility and total fertility. Mean absolute values of selection gradients ($\overline{|\beta|}$) are shown at the bottom as a measure of overall strength of selection. P values significant at < 0.05 are in bold.

	Early Experiment									Late Experiment								
	Male (outcross)			Male (total)			Female			Male (outcross)			Male (total)			Female		
	β	Pg	Pq	β	Pg	Pq	β	Pg	Pq	β	Pg	Pq	β	Pg	Pq	β	Pg	Pq
TL	0.38	0.14	**0.00**	0.23	0.32	0.05	-0.08	0.55	0.61	-0.05	0.69	0.76	0.06	0.64	0.67	0.10	0.52	0.51
TW	0.21	0.49	0.23	0.14	0.61	0.49	0.07	0.66	0.83	-0.08	0.61	0.65	0.01	0.94	0.98	0.19	0.32	0.38
PW	-0.77	**0.02**	**0.00**	-0.51	0.07	**0.04**	0.30	0.09	**0.05**	-0.03	0.87	0.80	0.03	0.89	0.86	-0.15	0.48	0.72
PL	0.72	**0.03**	**0.01**	0.43	0.16	0.12	-0.40	**0.04**	**0.02**	0.15	0.35	0.34	0.03	0.88	0.84	-0.01	0.95	0.94
FR	0.36	0.14	**0.01**	0.19	0.39	0.15	-0.11	0.40	0.13	-0.02	0.89	0.78	-0.11	0.47	0.39	-0.13	0.45	0.23
ST	-0.32	0.27	0.29	-0.14	0.59	0.93	0.07	0.66	0.22	-0.05	0.73	0.74	-0.06	0.74	0.81	0.00	1.00	0.92
AN	0.00	1.00	0.20	-0.13	0.57	0.20	0.06	0.66	0.28	**0.22**	**0.10**	0.07	0.18	0.20	0.17	0.04	0.82	0.32
NF	0.57	**0.03**	**0.00**	0.63	**0.01**	**0.00**	0.79	**0.00**	**0.00**	0.13	0.29	0.21	0.16	0.24	0.21	0.71	**0.00**	**0.00**
HT	0.41	0.11	0.14	0.36	0.13	0.09	-0.05	0.71	0.79	-0.09	0.48	0.44	0.11	0.42	0.39	0.23	0.13	0.08
$\overline{\lvert\beta\rvert}$	0.42	-	-	0.31	-	-	0.22	-	-	0.09	-	-	0.08	-	-	0.17	-	-

PL: petal length; PW: petal width; FR: number of petal fringes; TL: corolla tube length; TW: corolla tube width; ST: stigma exsertion; AN: anther exsertion; HT: display height; NF: number of flowers.

Table 4. Results of generalized linear multiple regressions through male and female functions during early and late flowering season of 2013 for *Silene stellata* near Mountain Lake Biological Station. P-values based on both Gaussian (Pg) and quasi-Poisson (Pq) models are shown next to the standardized linear regression coefficients (β). Gaussian models were performed using relative fitness as the response, while quasi-Poisson models were based on untransformed count data. Selection through male fitness was quantified using both outcrossing fertility and total fertility. Mean absolute values of selection gradients ($|\overline{\beta}|$) are shown at the bottom as a measure of overall strength of selection. P values significant at < 0.05 are in bold.

	Early Experiment									Late Experiment								
	Male (outcross)			Male (total)			Female			Male (outcross)			Male (total)			Female		
	β	Pg	Pq	β	Pg	Pq	β	Pg	Pq	β	Pg	Pq	β	Pg	Pq	β	Pg	Pq
TL	-0.13	0.20	0.28	-0.16	0.14	0.15	-0.10	0.25	0.12	-0.17	0.41	0.43	-0.16	0.43	0.44	-0.07	0.38	0.21
TW	-0.27	**0.01**	**0.01**	-0.11	0.32	0.19	-0.06	0.51	0.64	-0.17	0.46	0.39	-0.10	0.65	0.50	0.11	0.23	0.79
PW	-0.18	0.19	0.16	-0.08	0.60	0.63	0.24	**0.05**	**0.04**	0.20	0.46	0.60	0.10	0.71	0.85	0.00	1.00	0.68
PL	0.36	**0.02**	**0.03**	0.35	**0.03**	**0.05**	-0.24	0.06	**0.04**	0.30	0.26	0.31	0.34	0.20	0.22	0.00	0.97	0.77
FR	0.26	**0.01**	**0.02**	0.20	0.07	0.14	-0.02	0.86	0.92	-0.06	0.76	0.89	-0.06	0.76	0.88	0.03	0.72	0.60
ST	-0.06	0.66	0.63	-0.06	0.62	0.66	0.01	0.92	0.83	-0.03	0.90	0.92	-0.08	0.68	0.90	0.04	0.60	0.09
AN	0.06	0.67	0.62	0.12	0.39	0.44	0.06	0.60	0.52	0.15	0.44	0.53	0.11	0.57	0.63	0.09	0.22	0.23
NF	0.10	0.36	0.24	0.15	0.16	0.19	0.49	**0.00**	**0.00**	0.19	0.34	0.28	0.26	0.19	0.16	0.59	**0.00**	**0.00**
HT	0.03	0.73	0.78	0.08	0.41	0.35	0.02	0.85	0.55	0.08	0.70	0.87	0.09	0.65	0.83	0.08	0.34	0.16
$\lvert\overline{\beta}\rvert$	0.16	-	-	0.15	-	-	0.14	-	-	0.15	-	-	0.14	-	-	0.11	-	-

PL: petal length; PW: petal width; FR: number of petal fringes; TL: corolla tube length; TW: corolla tube width; ST: stigma exsertion; AN: anther exsertion; HT: display height; NF: number of flowers.

Table 5. AIC scores and deviance statistics for the five Analysis of Covariance (ANCOVA) models testing trait × sex, trait × pollinator and trait × sex × pollinator interactions for one population of *Silene stellata* near Mountain Lake Biological Station. Main effects of nine traits, sex and pollinator types were included in all models.

	2012		2013	
Model	AIC	Residual deviance (df)	AIC	Residual deviance (df)
Null Model	720.8	710.8 (229)	593.4	587.4 (217)
Model 1 (ME)	678.8	650.8 (220)	575.7	547.7 (215)
Model 2 (ME + TR × P)	694.1	648.1 (211)	587.2	541.2 (206)
Model 3 (ME + TR × S)	667.2	621.2 (211)	562.9	516.9 (197)
Model 4 (ME + TR × P + TR × S)	682.2	618.2 (202)	574.2	510.2 (197)
Model 5 (ME + TR × P + TR × S + TR × P × S)	684.4	600.4 (192)	586.8	502.8 (188)

ME: main effects; TR: trait; P: pollinator type; S: sex.

Table 6. ANCOVA results testing for differential selection on floral traits of *Silene stellata* between sexual functions in 2012 and 2013 near Mountain Lake Biological Station. Trait main effects represent total selection averaged over male and female reproductive success, and trait × sex interactions test for differences in selection through male vs. female functions. Plant was included as a random effect. Main effect of sex was also included in the model but is not shown for simplicity. Effects in bold are significant at $P < 0.05$.

	2012				2013			
Effect	SS	df	F ratio	P-value	SS	df	F ratio	P-value
TL	0.13	1	0.12	0.73	1.36	1	2.54	0.11
TW	0.05	1	0.04	0.84	0.70	1	1.31	0.25
PW	1.43	1	1.35	0.25	0.47	1	0.88	0.35
PL	1.89	1	1.78	0.18	0.50	1	0.94	0.34
FR	0.21	1	0.20	0.66	0.15	1	0.29	0.59
ST	0.30	1	0.28	0.60	0.07	1	0.12	0.73
AN	0.21	1	0.19	0.66	0.83	1	1.54	0.22
NF	**35.20**	**1**	**33.14**	**0.00**	**16.02**	**1**	**29.87**	**0.00**
HT	3.61	1	3.40	0.07	0.38	1	0.71	0.40
TL × Sex	1.45	1	1.37	0.25	0.09	1	0.17	0.68
TW × Sex	0.41	1	0.39	0.54	1.51	1	2.81	0.10
PW × Sex	**9.23**	**1**	**8.68**	**0.00**	0.17	1	0.31	0.58
PL × Sex	**14.39**	**1**	**13.55**	**0.00**	**3.98**	**1**	**7.43**	**0.01**
FR × Sex	**5.48**	**1**	**5.16**	**0.03**	0.14	1	0.27	0.61
ST × Sex	1.40	1	1.32	0.25	0.15	1	0.27	0.60
AN × Sex	0.01	1	0.01	0.93	0.02	1	0.03	0.87
NF × Sex	2.98	1	2.81	0.10	7.47	1	13.92	0.00
HT × Sex	0.12	1	0.11	0.74	0.01	1	0.01	0.91

PL: petal length; PW: petal width; FR: number of petal fringes; TL: corolla tube length; TW: corolla tube width; ST: stigma exsertion; AN: anther exsertion; HT: display height; NF: number of flowers

Table 7. Results of generalized linear multiple regressions through male and female functions using data pooled across early and late experiments for 2012 and 2013 for *Silene stellata* near Mountain Lake Biological Station. Standardized linear regression coefficients (β) and P-values based on Gaussian multiple regressions are shown. Selection through male function was quantified using both outcrossing fertility and total fertility. Mean absolute values of selection gradients ($\overline{|\beta|}$) are shown at the bottom as a measure of overall strength of selection. P values significant at < 0.10 are in bold.

	2012						2013					
	Male (outcross)		Male (total)		Female		Male (outcross)		Male (total)		Female	
	β	P-value	β	P-value	β	P-value	β	P-value	β	P-value	β	P-value
TL	0.14	0.32	0.10	0.45	-0.07	0.50	-0.12	0.27	-0.15	0.16	-0.08	0.17
TW	-0.07	0.70	-0.05	0.76	0.07	0.54	-0.18	0.16	-0.07	0.54	0.05	0.50
PW	-0.40	**0.04**	-0.27	0.11	0.17	0.18	0.03	0.83	0.03	0.86	0.14	**0.07**
PL	0.44	**0.02**	0.27	0.13	-0.22	**0.09**	0.30	**0.05**	0.35	**0.02**	-0.13	**0.10**
FR	0.22	0.13	0.09	0.48	-0.14	0.16	0.05	0.62	0.05	0.64	-0.01	0.88
ST	-0.06	0.69	0.03	0.86	0.05	0.65	-0.03	0.78	-0.08	0.52	0.04	0.51
AN	0.01	0.94	-0.08	0.56	0.03	0.79	0.06	0.59	0.10	0.38	0.05	0.44
NF	0.35	**0.01**	0.42	**0.00**	0.62	**0.00**	0.14	0.23	0.21	**0.06**	0.55	**0.00**
HT	0.13	0.36	0.20	0.12	0.13	0.18	0.04	0.69	0.09	0.40	0.03	0.59
$\overline{\lvert\beta\rvert}$	0.20	-	0.17	-	0.17	-	0.11	-	0.12	-	0.12	-

PL: petal length; PW: petal width; FR: number of petal fringes; TL: corolla tube length; TW: corolla tube width; ST: stigma exsertion; AN: anther exsertion; HT: display height; NF: number of flowers.

Table 8. Results of multiple regressions testing *Hadena ectypa* oviposition preference for floral traits of *Silene stellata.* Trait values were z-transformed and mean number of eggs per flower was used as the response variable in the Gaussian multiple regression models. Regression coefficients significant at $P < 0.05$ are in bold.

	2012		2013	
Trait	Coefficient	P-value	Coefficient	P-value
TL	0.05	0.72	0.15	0.10
TW	0.21	0.17	0.00	0.99
PW	-0.01	0.93	0.11	0.37
PL	-0.06	0.72	-0.04	0.78
FR	0.05	0.68	0.01	0.87
ST	0.12	0.38	0.06	0.56
AN	-0.17	0.16	0.03	0.78
NF	-0.31	**0.02**	-0.22	**0.02**
HT	-0.01	0.93	-0.02	0.80

PL: petal length; PW: petal width; FR: number of petal fringes; TL: corolla tube length; TW: corolla tube width; ST: stigma exsertion; AN: anther exsertion; HT: display height; NF: number of flowers.

Figure 1. Phenological data of moth and egg densities of 2012 for one population of *Silene stellata* near Mountain Lake Biological Station. Moth density was calculated as the number of *Hadena ectypa* or copollinating moths per flower × 100. Egg density was calculated as the mean number of eggs per flower. CP: copollinators; HE: *Hadena ectypa*.

Figure 2. Phenological data of moth and egg densities of 2013 for one population of *Silene stellata* near Mountain Lake Biological Station. Moth density was calculated as the number of *Hadena ectypa* or copollinating moths per flower × 100. Egg density was calculated as the mean number of eggs per flower. CP: copollinators; HE: *Hadena ectypa*.

Figure 3. Diagram of *Silene stellata* floral traits measured. (A) PL: petal length, PW: petal width, FR: number of petal fringes, TW: corolla tube width. (B) A male-phase flower. TL: corolla tube length, AN: anther exsertion. (C) A female-phase flower. ST: stigma exsertion. Scale bars = 5 mm.

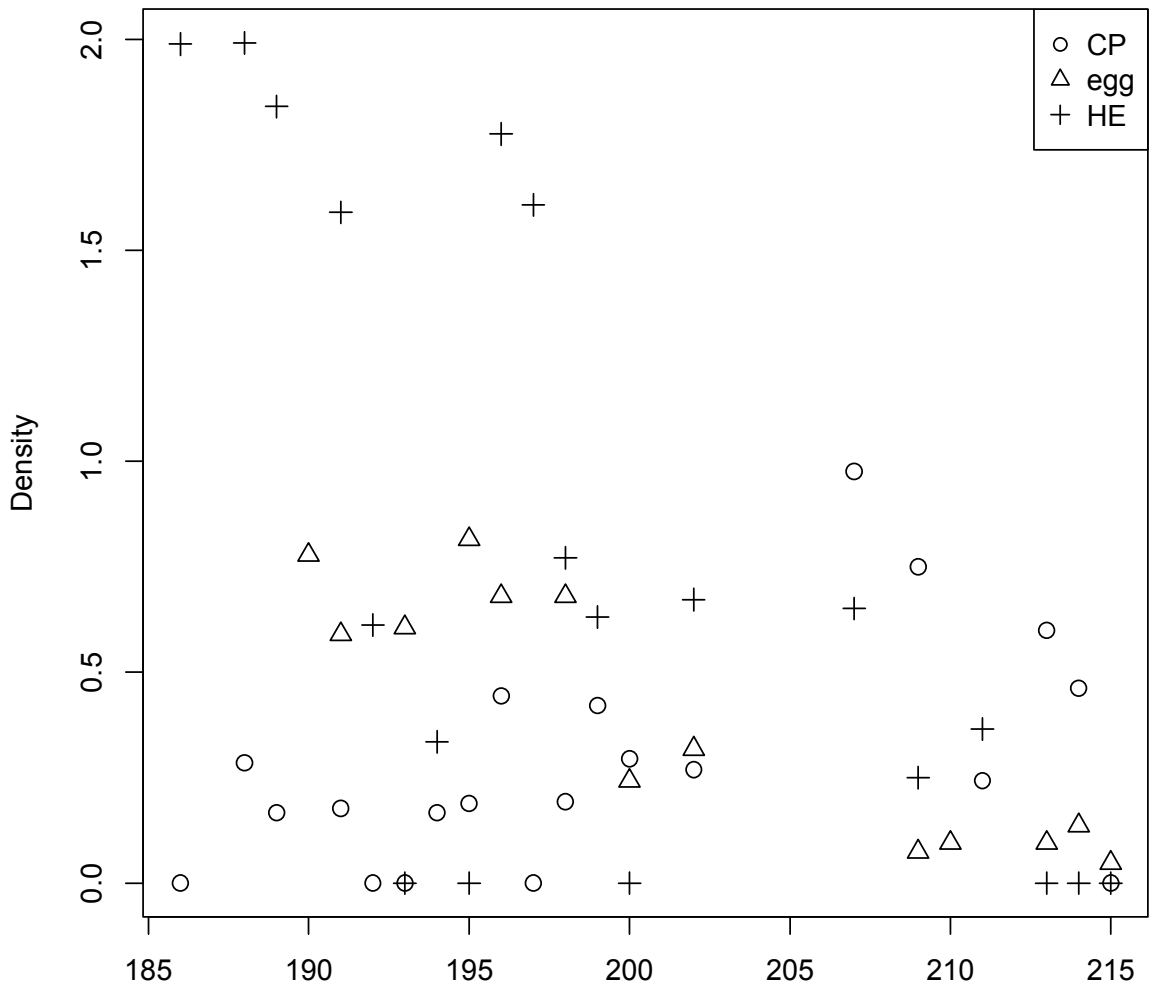


FIG. 1

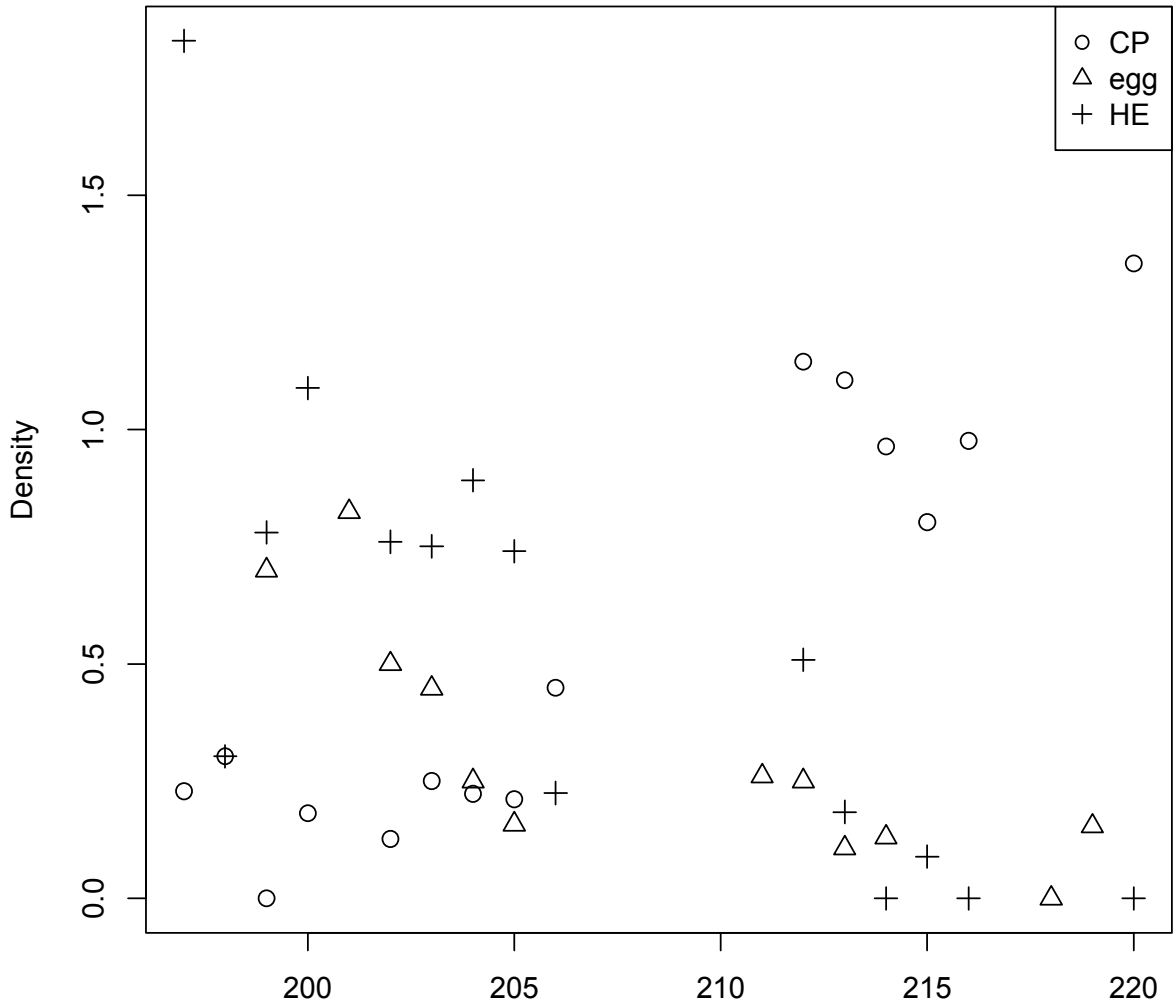


FIG. 2

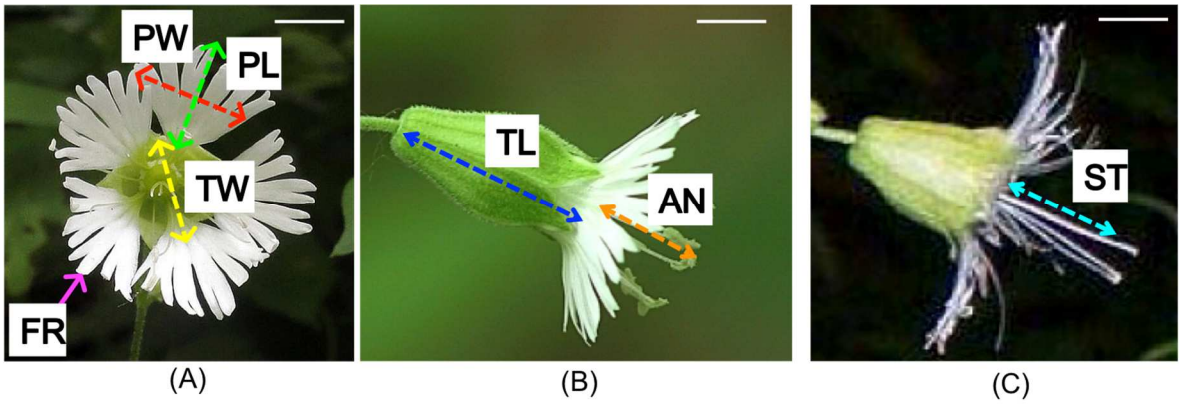


FIG. 3

Supplemental table 1. Results of Analysis of Covariance (ANCOVA) testing for trait × sex, trait × pollinator, and trait × sex × pollinator interactions using data pooled across years for *Silene stellata* near Mountain Lake Biological Station. Plant was included as a random effect. Male and female fitness scores were scaled to mean fitness of a particular pollinator-by-sex combination such that the main effects of sex and pollinator and sex × pollinator interactions were zero. Pollinator, sex and year main effects were also included in the model. Effects in bold are significant at $P < 0.05$.

Effect	SS	df	F ratio	P-value
TL	0.01	1.00	0.01	0.91
TW	0.19	1.00	0.25	0.62
PW	1.46	1.00	1.89	0.17
PL	2.14	1.00	2.78	0.10
FR	**3.10**	**1.00**	**4.02**	**0.05**
ST	0.50	1.00	0.65	0.42
AN	0.23	1.00	0.29	0.59
NF	**12.02**	**1.00**	**15.60**	**0.00**
HT	0.77	1.00	0.99	0.32
Pollinator	0.00	1.00	0.00	1.00
Sex	0.00	1.00	0.00	1.00
Year	0.00	1.00	0.00	1.00
TL × Pollinator	0.01	1.00	0.01	0.91
TW × Pollinator	0.20	1.00	0.26	0.61
PW × Pollinator	0.98	1.00	1.27	0.26
PL × Pollinator	0.94	1.00	1.21	0.27
FR × Pollinator	**3.20**	**1.00**	**4.15**	**0.04**
ST × Pollinator	0.31	1.00	0.40	0.53
AN × Pollinator	0.84	1.00	1.09	0.30
NF × Pollinator	1.47	1.00	1.90	0.17

HT × Pollinator	0.19	1.00	0.25	0.62
TL × Sex	1.57	1.00	2.04	0.15
TW × Sex	0.03	1.00	0.03	0.85
PW × Sex	**14.49**	**1.00**	**18.80**	**0.00**
PL × Sex	**14.05**	**1.00**	**18.23**	**0.00**
FR × Sex	**6.52**	**1.00**	**8.46**	**0.00**
ST × Sex	1.43	1.00	1.85	0.18
AN × Sex	0.14	1.00	0.19	0.67
NF × Sex	0.08	1.00	0.11	0.74
HT × Sex	**2.95**	**1.00**	**3.83**	**0.05**
E × Sex	0.00	1.00	0.00	1.00
TL × Pollinator × Sex	1.11	1.00	1.44	0.23
TW × Pollinator × Sex	0.42	1.00	0.55	0.46
PW × Pollinator × Sex	**11.13**	**1.00**	**14.44**	**0.00**
PL × Pollinator × Sex	**7.47**	**1.00**	**9.69**	**0.00**
FR × Pollinator × Sex	**4.94**	**1.00**	**6.41**	**0.01**
ST × Pollinator × Sex	0.79	1.00	1.03	0.31
AN × Pollinator × Sex	0.34	1.00	0.44	0.51
NF × Pollinator × Sex	2.43	1.00	3.15	0.08
HT × Pollinator × Sex	**3.02**	**1.00**	**3.92**	**0.05**

PL: petal length; PW: petal width; FR: number of petal fringes; TL: corolla tube length; TW: corolla tube width; ST: stigma exsertion; AN: anther exsertion; HT: display height; NF: number of flowers.

Chapter 4: Quantification of genetic variation of floral design of the tetraploid plant *Silene stellata* using the pedigree-free animal model.

Abstract

The amount of genetic variation of floral traits and the degree to which they are genetically correlated are important parameters for floral evolutionary studies. Estimates of these parameters can reveal the effect of historical selection relative to neutral processes such as mutation and drift, and also allow us to predict the evolutionary trajectory of a population under various selection scenarios. We assess the heritabilities and genetic correlations of nine traits of the native N. American perennial plant *Silene stellata* (Caryophyllaceae) in the field. In one of the first attempts for plants, we estimated the genetic parameters using the "animal model" approach based on the phenotypes of 227 *S. stellata* individuals from a natural population. Here, we explored the feasibility of applying the pedigree-free animal model to plant populations using a genealogy reconstructed from 11 highly variable microsatellite loci in place of the known pedigree. We found significant heritabilities in five out of nine traits. The level of heritability was intermediate (0.027 – 0.441). We also found prevalent positive genetic correlations between floral traits. Our results suggest that *S. stellata* is capable of responding to phenotypic selection on its floral design, while the abundant genetic correlations could also pose certain constraints on trait divergence. Furthermore, our successful use of the animal model approach using individuals of inferred relationship growing in their native environment demonstrates the utility and feasibility of marker-based approaches for estimating genetic parameters, bypassing the necessity of

employing controlled crosses and growing multiple generations in an artificial environment.

Introduction

An objective in evolutionary biology is to predict the phenotypic changes of populations under the force of selection. This requires, along with the estimate of selection pressures, detailed understanding of the underlying genetic variation of quantitative traits as well as genetic correlations between traits, as illustrated by the multivariate breeder's equation (Lande and Arnold 1983). Furthermore, the pattern of existing genetic variation also reveals the efficacy of historical selection relative to genetic drift, mutation and migration (Lynch and Walsh 1998).

Since the application of the breeder's equation to evolutionary studies (Lande and Arnold 1983), extensive efforts have been dedicated to studying the levels of genetic variation in morphological and life history characters in plants (e.g., Ashman and Majetic 2006). Because floral diversity is believed to reflect, in part, pollinator mediated selection (Fenster et al. 2004), and mating system evolution is linked to floral evolution (Barrett 1998), documentation of within population genetic variation of floral traits has been a historic focus. A survey by Ashman and Majetic (2006) based on studies conducted mostly in controlled environments found an average h^2 of 0.39. Although it seems that there is considerable standing genetic variation in floral traits to allow evolutionary response to pollinator-mediated selection (Ashman and Majetic 2006) and other forces such as herbivores (Strauss and Whittall 2006), genetic parameters estimated in

controlled environments should be interpreted cautiously since estimation of heritability in controlled environments could be potentially biased upward due to the lower environmental effects as well as G×E interactions (Coyne and Beecham 1987; Conner et al. 2003). Genetic parameters estimated in the wild, on the other hand, take in to account environmental variables affecting natural populations, therefore are more reliable in predicting microevolutionary changes together with data of selection gradients estimated in natural environments. For instance, Grant and Grant (1995) correctly predicted the response in body and beak size traits of Darwin's ground finch (*Geospiza fortis*) based on selection gradients and genetic parameters estimated in the field using parent-offspring regression.

Traditional methods of heritability estimation rely on the covariation of breeding values (genetic merits) between close relatives and usually require construction of experimental populations with uniform family structures, such as parent-offspring, full sibs, half-sibs, etc. (Falconer and Mackay 1996). A large sample size is also required to ensure statistical power (Mitchell-Olds and Rutledge 1986). The cost and labor and most importantly, time of setting up such a breeding program are significant obstacles to the study of genetic variation in wild plant populations, especially for long-lived organisms.

Recently, a restricted maximum likelihood (REML) approach termed the "animal model" has been applied to investigate genetic bases of complex traits in wild populations (Kruuk 2004; Wilson et al. 2010). The animal model is more flexible in that it does not require a fixed family structure in the study population and is able to take into account all relationships in any pedigree. The animal model is therefore expected to provide more statistical power than close-kin comparisons (Kruuk 2004). It is also less

susceptible to confounding effects such as inbreeding, selection and shared environment (Kruuk and Hadfield 2007), and is more robust to unbalanced datasets than parent-offspring regression approaches (Lynch et al. 1998; Kesson et al. 2008). To date, the animal model has mostly been applied to animal systems under long-term population study. For example, applying the animal model to the red deer (*Cervus elaphus*) population on the Isle of Rum, Kruuk et al. (2002) were able to predicate the evolutionary response in antler size, a trait under sexual selection, by combining field-estimated genetic parameters and selection gradients.

Given all these advantages, one limitation of the animal model in its application to field studies is the requirement of a fully known pedigree, which is generally very difficult to acquire in natural populations, especially for organisms with complex life history and mating systems. Therefore, despite its success in animal and plant breeding, application of the animal model to evolutionary studies has been relatively rare and mostly has been restricted to bird and large mammal populations under long term monitoring, and whose behavior allow the inference of relatedness between individuals (Milner et al. 2000, Kruuk 2004, Kesson et al. 2008, Akesson et al. 2008). In contrast, the availability of highly variable molecular marker and various statistical methods now makes it possible to implement the animal model with genealogies reconstructed based on individual genotypes (Frentiu et al. 2008; Blonk et al. 2010; Frère et al. 2010). This approach has the potential to expand the range of application of the animal model to wild populations and has been shown to perform well in several animal systems (Frentiu et al. 2008; Blonk et al. 2010; Frère et al. 2010).

Here, using the animal model, we determine the heritabilities as well as genetic correlations of traits in a natural population of the perennial plant *Silene stellata*. We construct a genealogy based on 11 highly variable microsatellite loci. *Silene stellata* has a complex interaction with its obligate pollinating seed predator, the nocturnal moth *Hadena ectypa* (Noctuideae) as well as a number of generalist moths. *Hadena ectypa* has simultaneously a positive effect as a pollinator and a negative effect as a seed predator in the larval stage.

There are mainly three ways the quantification of the genetic basis of *S. stellata* morphological traits can bring insight into the evolutionary biology of the *Silene-Hadena* interaction. First, negative selection mediated by the strong seed predation of the *H. ectypa* larvae on the *S. stellata* floral design is likely to be present for female fitness as shown by previous work (Kula et al. 2013, Reynolds et al. unpublished, chapter 3). It is therefore imperative to examine if the *S. stellata* populations retain sufficient additive genetic variation to evolve an "escape route" from *H. ectypa* or nocturnal pollination syndromes in general (Kephart et al. 2006). Secondly, selection can potentially be exerted upon the floral design through the positive interaction of pollination and negative interaction of seed predation by the *H. ectypa* larvae, so it is important to test whether conflicting selection could be present indirectly through genetic correlation between traits (Strauss and Whittall 2006). Finally, the net outcome of the *Silene-Hadena* interaction has been shown to be variable depending on the pollination service provided by the copollinators, ranging from parasitic to possibly mutualistic (Kephart et al. 2006; Reynolds et al. 2012). This suggests that phenotypic selection imposed by the pollinator community could be temporally fluctuating. We can use low observed heritability to

reject the hypothesis that fluctuating selection is an important force maintaining genetic variation (Robertson 1956; Yampolsky et al. 1994; Kondrashov and Yampolsky 1996). Therefore, in this paper, we quantify the genetic basis of nine traits of *S. stellata* in order to better understand the effect of historical selection on the *S. stellata* floral design and to enable us to make predictions about microevolutionary changes under complex selection scenarios in nature.

Methods

Study population

We measured *S. stellata* plants in four cohorts in 2012 and 2013. In each year, two cohorts of flowering plants were measured in the course of a week during both the early and late flowering season with a one-week separation between experimental periods. We sampled different plants for each cohort and in each year to avoid pseudo-replication, yielding a total number of 227 plants (2012 early, N = 59; 2012 late, N = 58; 2013 early, N = 55; 2014 late, N = 55). The within-year cohort sampling reflects the change across the phenology with adult *H. ectypa*'s being prevalent early in the season, but of much lower frequency as a pollinator later in the season. Our sampling of genotypes to quantify patterns of genetic variation thus takes into account very different selection pressures that early and late flowering plants may experience. The same plants were used in a two-year field experiment aimed at quantifying phenotypic selection on the *S. stellata* floral design. All study plants were within a 20 m × 20 m deer exclosure constructed at the Meadow population near Mountain Lake Biological Station (37.348296°, -80.544301°,

elevation ≈ 1,100–1,300 m). The exclosure was completely porous to the typical pollinators of *S. stellata* (personal observation).

In contrast to the predominant diploidy of *Silene* species distributed in the Old World, the vast majority of N. American *Silene* have been shown to be polyploids, including tetra-, hexa-, and octoploids, with tetraploids being the most common type (Popp and Oxelman 2007). The N. American polyploid *Silene* species have at least two independent origins (Popp and Oxelman 2007). Phylogenetic relationships within the clade containing *S. stellata* are poorly resolved, possibly due to rapid evolution, recombination among homoeologues, and homoplasy (Popp and Oxelman 2007). The status of (auto- vs. allo-) tetraploidy of *S. stellata* is not well understood. However, the maximum number of alleles amplified per individual per locus was consistently four across all 11 markers in this study and we found no evidence of fixed heterozygotes, supporting a hypothesis of autotetraploidy (Parisod et al. 2010).

Phenotypic traits

We quantified the pattern of genetic variation and covariation for nine floral characters: 1) corolla tube length (TL); 2) corolla tube width (TW); 3) largest petal length (PL); 4) largest petal width (PW); 5) number of fringes on the distal margin of the petal (FR); 6) Anther exsertion (AN); 7) stigma exsertion (ST). We measured the distance from the nectary at the base of the flower to the tip of the anther or the stigma and calculated AN and ST as the difference between nectary-anther or nectary-stigma distance and corolla tube length. All floral traits except ST were measured on flowers during the first day of opening (the male phase). Stigma measurements were taken in the first day of the female phase. We counted fringe number (FR) on a randomly chosen petal in which we

measured the other six floral traits. Additionally, we recorded the total number of flowers (NF) and floral display height (HT). Measurements were carried out with calipers (0.1 mm) during the course of one week on each plant for PL, PW, FR, TL, TW, ST, AN. Display height was measured as the height of the highest flower to the nearest cm using a meter stick, while NF was quantified by counting the number of flowers open throughout the experimental period. On average five flowers per plant were measured for floral traits (total number of flowers measured, mean number measured per plant: 2012 early, N = 280, 4.75; 2012 late, N = 239, 4.12; 2013 early, N = 294, 5.35; 2014 late, N = 281, 5.11).

Genotyping and genealogy reconstruction

DNA was extracted using the Autogen Plant Kit, following the manufacturer's protocol. Plants were genotyped on 11 novel microsatellite loci designed for *S. stellata* (Zhou et al. 2016*b*). Individual genotypes were visualized and manually scored using GeneMapper V3.7 software (Applied Biosystems).

There are two main approaches to replace the known pedigree in the animal model. First, one can construct a matrix, **A**, consisting of relatedness coefficients that are independently estimated for all pairs of individuals. Since the relatedness coefficients are estimated independently for each pair of individuals, compatibility between dyads is not guaranteed (e.g. in examining the relationships between three individuals, A, B and C, the pairs A–B and A–C might be inferred as full-sibs, while the pair B–C might be inferred as half- or non-sib). This incompatibility can lead to the non-positive-definiteness of **A**, resulting in its failure to be inverted for the subsequent REML analysis (Frentiu et al. 2008). Therefore, we implemented the simulated annealing approach using the program COLONY (Jones and Wang 2010) to reconstruct a genealogy containing all study plants

that ensured statistical consistency. COLONY uses the full-pedigree likelihood approach by maximizing the likelihood of the entire pedigree structure rather than individual relationships. Therefore it uses genotype data in a more efficient way and also avoids the troublesome compatibility issue (Jones and Wang 2010). Since *S. stellata* is a perennial plant, we were prone to sample parent-offspring dyads in the study population in addition to full-sib and half-sib dyads. Therefore we allow COLONY to simultaneously infer parentage and sibship between the study plants, in order to account for the extended age structure that is likely to be present in the study *S. stellata* population (Dudash and Fenster unpublished data). Using COLONY, we generated a genealogy in which all pairs of individuals were classified as full sibs, half sibs, parent-offspring, or unrelated. Since less related pairs of individuals will not be reflected in the estimated genealogy, some information in the data is inherently lost. However, the advantage of genealogy reconstruction is that all pairwise relationships are inherently consistent and the pedigree data format is compatible with all REML software.

Given that *S. stellata* is a tetraploid, the underlying genotypes of partial heterozygotes cannot be readily resolved (for example, an individual showing two alleles A, B on a given locus can have AAAB, AABB or ABBB as the underlying genotype). Additionally, most programs of genealogy reconstruction do not support polyploid data. In the face of these technical difficulties, we transformed the tetraploid genotypes following the protocol described in Wang and Scribner (2014) for application in polyploids. Specifically, we converted the tetraploid genotypes at a codominant microsatellite locus with k alleles to diploid phenotypes at k dominant “loci”, following the method of Rodzen et al. (2004). Each pseudo-dominant locus thus has two

phenotypes: the dominant phenotype 1, corresponding to the underlying genotypes {1,1} and {1,0} and the recessive phenotype corresponding to the genotype {0,0}. For example, for a locus with four alleles (A1, A2, A3, A4), an individual with alleles A1, A2, will be coded as {1, 1, 0, 0} at four pseudo-diploid loci, and an individual with alleles A1, A2, A3 will be coded as {1, 1, 1, 0}, and so forth (See Supplemental Table 1 for a detailed example). This approach has been demonstrated to accurately infer sibship, parentage and selfing rate from a typical set of microsatellite markers (Wang and Scribner 2014).

To assess the reliability of the genealogy reconstructed using the above procedure, we also generated a matrix consisting of independent estimates of pairwise relatedness coefficients, r, using the package POLYRELATEDNESS 1.6 (Huang et al. 2014), designed for autopolyploids. POLYRELATEDNESS uses a maximum likelihood procedure to estimate the vector $\Delta = [\Delta_1\ \Delta_2\ \Delta_3\ \Delta_4]$, for every pairwise comparison, where Δ_i is the probability that two individuals share i alleles that are Identical-By-Descent (IBD). The relatedness coefficient r is then calculated as. We converted the genealogy generated by COLONY to a relatedness matrix using the statistical package "kinship2" (Therneau and Sinnwell 2015) in R (R Core Team 2016). We then compared the two matrices generated by POLYRELATEDNESS and COLONY by running a mantel test in R with 9999 permutations and found a significant correlation (Pearson correlation coefficient $r = 0.665$, $p < 0.001$).

Animal model analysis

Univariate variance component analysis was carried out in the REML program Wombat (Meyer 2007). We fit the following repeated-measure animal model for the seven floral

traits using data pooled across years with year and season as fixed effects: $y = Xb + Z_a a + Z_c c + e$, where y is the vector of observed phenotypic values, b is the vector containing the main effects of year (2012 vs. 2013) and season (early vs. late), a is the vector containing individual additive genetic effects, c represents the vector of permanent environmental effects, and e contains the residuals. X, Z_a, and Z_c are design matrices relating appropriate fixed and random effects to individual records. The multiple measurements of a floral trait on a given plant were grouped under the same permanent environmental effect potentially including micro-environmental, maternal, dominance, and epistatic effects on each individual. Permanent environmental effects were not included in the model for number of flowers (NF) and display height (HT), for which only one record was available per plant. The total phenotypic variance V_P was therefore partitioned into additive genetic variance (V_A), variance due to permanent environmental effect (V_{PE}), and residual variance (V_R). Narrow-sense heritabilities were then calculated using the estimated additive genetic variance as. We also reported the repeatability estimates for the seven floral traits as. For comparative purposes, we also report the coefficient of variation (CV), i.e., 100 × additive genetic variance divided by the phenotypic mean.

We first fit separate models for 2012 and 2013 without the year effect. We then compared the h^2 estimates across years and found no significant difference for any trait (Supplemental Table 2; Figure 1). Therefore, in the subsequent univariate analyses we pooled the data across years and employed the full model with year as a fixed effect. We specified Wombat to sample 100000 data points from the multivariate normal distribution

of ML estimates generated at convergence, to approximate the standard errors for the parameter estimates.

Genetic correlations

Genetic correlations, were estimated using the multivariate version of the animal model described above. The model included only traits for which we observed significant additive genetic components in previous univariate analyses, since is ill-defined if one trait has heritability equal to zero because the genetic correlation between trait x and y is defined as: $r_A = \frac{Cov_{A(xy)}}{\sqrt{V_{A(x)}V_{A(y)}}}$, where $Cov_{A(xy)}$ is the genetic covariance (Lynch and Walsh 1998; Coltman et al. 2005). Additionally, we also report phenotypic correlations between pairs of traits based on the outputs of Wombat. Similar to univariate analyses, we first fit the multivariate animal model for each year independently, then compared the estimates between years. Since no significant differences occurred between years (Supplemental Table 3; Figure 2), we then pooled the data across years and performed the multivariate animal model using the full model with year as a fixed effect.

Testing possible correlation of environmental effects between relatives

Since previous work has shown that pollen dispersal distance between plants is as low as 1-2 m (Reynolds et al. 2009, chapter 1), and seeds of *S. stellata* mainly disperse passively through gravity, closely related plants could experience common environmental effects due to spatial autocorrelation, which could potentially inflate our genetic component estimates. To rule out this possibility, we compared the relatedness matrix A generated using COLONY with the matrix D which contained pairwise Euclidean distances calculated based on individual GPS coordinates, by running a mantel test with 9999

permutations in R (R Core Team 2016). No significant correlation was found between the two matrices (Pearson correlation coefficient r = -0.048, $p > 0.999$).

Statistics

We assessed the significance levels of individual parameter estimates as well as comparison of estimates across years by calculating the *z* score, equal to the difference between two estimates divided by the square root of the sum of the squared standard errors. For the purpose of testing significance of single estimates against zero, the formula is reduced to the ratio between the estimate and its standard error. *P*-values of the *z* scores were calculated from a large sample standard normal distribution. We also quantified the association in estimates of h^2, r_A between years using Pearson's product-moment correlation (*r*). Additionally, a Mantel test with 9999 permutations was used to compare the two r_A matrices for 2012 and 2013. All comparisons were performed in R (R Core Team 2016).

Results

Heritability and variance components

Using the pooled dataset, we found five floral traits (PL, FR, TL, TW, and PW) with significant h^2 ($p < 0.05$), while the h^2 of anther exsertion (AN) was marginally significant ($p = 0.085$) and was included in subsequent multivariate analyses of genetic correlations. h^2 values ranged from 0 (NF, $p > 0.99$) to 0.441 (PL, $p < 0.001$). Genetic coefficients of

variation (CV) ranged between 0.266 (NF) and 14.049. (PL). Repeatabilities were significant in all cases ($p < 0.05$) and ranged from 0.391 (ST) to 0.885 (PL) (Table 1).

Genetic correlations

Genetic correlations calculated using the pooled dataset were positive in 14 of 15 comparisons and significant in nine of them (mean $r_A = 0.236 \pm 0.295$ SE, Table 2). All significant r_A's were positive and ranged between 0.324 and 0.972. The highest r_A was TL vs. AN ($r_A = 0.972 \pm 0.175$ SE, $p < 0.001$), followed by PL vs. PW ($r_A = 0.944 \pm 0.260$ SE, $p < 0.001$). Petal fringe (FR) was only significantly correlated with PW ($h^2 = 0.324 \pm 0.161$ SE, $p < 0.05$).

Similar to genetic correlations, values of phenotypic correlations r_p were positive except for TL vs. TW ($r_P = -0.208 \pm 0.053$ SE, $p < 0.001$) (Table 2). Mean $r_p = 0.2538 \pm 0.202$ SD. The only insignificant r_p was TL vs. FR ($r_P = 0.08 \pm 0.055$ SE, $p > 0.05$). The average squared genetic correlation (r^2_A) was 0.28, while the average squared phenotypic correlation (r^2_P) was 0.10 (Cheverud 1988). A Mantel test with 999 permutations revealed significant correlation between the genetic and phenotypic correlation matrices (Pearson correlation coefficient $r = 0.91$, $p < 0.001$).

Discussion

Genealogy reconstruction

Although statistically more powerful and flexible than traditional methods such as parent-offspring regression, the requirement of a known pedigree has impeded the application of

the animal model to natural plant populations. There have been a number of studies that attempted to replace the known pedigree with one reconstructed from molecular genotypes (Frentiu et al. 2008, Blonk et al. 2010, Frère et al. 2010). These results generally support the efficacy of the pedigree-free approach in estimating genetic components. However, to our knowledge, there has been no such application to wild plant populations. Here we reconstructed a genealogy for 227 *S. stellata* individuals based on 11 highly variable microsatellite loci and observed prevalent genetic variation as well as covariation in the floral traits of *S. stellata*.

One major challenge in using molecular genealogy in an animal model analysis is the statistical error in the inference of pairwise relationship due to the limitation of genetic markers and the genealogy reconstruction algorithm, because the pedigree is assumed by the model to be known without error. Little empirical work has been conducted to examine how erroneous relationship inference affects the animal model. However, we used a microsatellite marker set that was highly polymorphic (mean number of alleles per locus = 22.6). Additionally, we observed a fair range of relatedness including unrelated, half-sib, full-sib and parent-offspring in the reconstructed genealogy. The high resolution of our marker set and the variation in relatedness may help to minimize the effect of erroneous relationships on our inference of genetic parameters (Ritland 1996; Frentiu et al. 2008).

Genetic variation

We observed significant heritabilities, h^2 in five out of nine traits of *S. stellata*. Additionally, anther exsertion (AN) was marginally significant ($h^2 = 0.257 \pm 0.149$ SD, $p = 0.085$). The h^2's were intermediate (0.027–0.441) for all the remaining floral traits.

Among all floral traits, stigma exsertion (ST) had the lowest heritability ($h^2 = 0.027 \pm 0.131$ SD, $p > 0.10$). One likely explanation for this low h^2 other than strong historical selection, is that the protandrous *S. stellata* flowers transition to the female phase after the one-day male-phase through slow extension of the style. Thus, although we made all measurements of ST on flowers during the first day of the female stage, the exact time of measurements and possible asynchrony in growth rates of the styles could introduce additional environmental variation. Alternatively, we observed strong inbreeding depression and significant positive selection for anther-stigma separation in the study *S. stellata* population (Chapter 1). Therefore, strong historical selection for the avoidance of inbreeding depression could also lead to the low observed h^2 and CV_A for stigma and anther exertion. We did not find significant h^2 in display height (HT) and number of flowers (NF). One likely cause for the lack of genetic components in NF and HT could be that *S. stellata* is a long-lived perennial, and phenotypic variation in these two traits mainly reflects variation in plant age and local microhabitat, rather than the underlying genetic factors.

Our mean estimate of h^2 over all nine traits was 0.27, while mean h^2 of the seven floral ones was 0.32. These estimates were lower than the mean of 0.39 reported by Ashman and Majetic (2006). This is not surprising, as 66% percent of the studies surveyed by Ashman and Majetic (2006) were conducted in controlled environments and high environmental variance in field conditions might contribute to our lower observed h^2.

Genetic correlations

We found significant genetic correlations, r_A in nine out of 15 pairwise comparisons. We found a mean r_A of 0.45, which is higher than Ashman and Majetic (2006) (Mean±ST = 0.33±0.03). Our inclusion of only traits with significant h^2 may have contributed to the high average r_A estimates. Following Cheverud (1988), we compared the phenotypic and genetic correlation matrices using the average squared correlation coefficients. The average squared genetic correlation was 0.28, while the average squared phenotypic correlation was 0.10. This difference is very close to the mean difference of 0.20 reported by Cheverud (1988), and may be caused by sampling error given the limitation of a pedigree-free animal model. Alternatively, the observed differences could be caused by significant environmental variation in the natural environment (Willis et al. 1991), since the majority of cases studied by Cheverud (1988) were conducted in laboratory or agricultural environments.

The significant r_A's were uniformly positive. This suggests that the floral design of *S. stellata* could potentially respond uniformly to natural selection for flower size. On the other hand, divergence of character states through selection may be constrained along axes determined by the abundant genetic correlations (Falconer 1981). Specifically, genetic correlations between traits associated with pollinator attraction (PL, PW, FR) (Bell 1985; Ashman and Majetic 2006; Mitchell et al. 2015) and traits associated with pollen transfer (TL, TW, AN, ST) (Nilsson 1988; Campbell et al. 1991, 1996; Ashman and Majetic 2006; Reynolds et al. 2010) were significant in six of nine cases. These positive genetic correlations could hamper independent evolution of different functional modules under potentially opposing selection pressures by various selective agents (e.g.,

mutualists vs. antagonists) and through the male vs. female function of the hermaphroditic flower (Strauss and Whittall 2006; Steven et al. 2007). In contrast, the lack of genetic correlation between certain pairs of floral traits could provide additional minor axes for divergence of character states. For example, the r_A between corolla tube length (TL) and width (TW) is close to zero ($r_A = -0.062 \pm 0.138$ ST, $p > 0.10$) while both traits had significant h^2. This indicates that these two dimensions of the *S. stellata* corolla tube could potentially respond independently to opposing selection pressures.

Conclusions

We observed substantial neutral genetic variation in three local *S. stellata* populations in a previous study (Chapter 2). The assessment of genetic variation in morphological traits here seems to be concordant with this previous finding although there is generally a weak relationship between levels of variation of neutral markers and quantitative traits (Reed et al. 2002) An earlier multi-year study of the same *S. stellata* population as this study showed that certain floral traits were under phenotypic selection significant only in certain years (Reynolds et al. unpublished). This implies that the standing genetic variation observed could have been maintained through the temporal variability in selection pressures documented. Additionally, the genetic variation could also have been maintained by the conflict between the selection to avoid *H. ectypa* predation and the selection to increase pollinator attraction.

While G×E interactions could also be responsible for the maintenance of genetic variation (Gillespie and Turelli 1989), the high similarities in h^2 and r_A across years do not seem to support this explanation. On the other hand, our results suggest the *S. stellata* population is capable of responding to directional selection, whereas the genetic

correlation between traits could also impose evolutionary constraints in certain multivariate selection scenarios. Our study represents an attempt in using the cost-efficient pedigree-free animal model in wild plant populations and we hope this study will facilitate future applications in evolutionary studies of plants.

Table 1. Heritabilities (h^2) and repeatabilities (r^2) of nine morphological traits of *Silene stellata*, with corresponding standard errors (SE), *z* scores (*z*), and P-values (*p*). Parameters were estimated with data pooled across two years including year as a fixed effect, using the univariate animal model. CV_A: coefficient of variation of additive genetic variance.

Trait	h^2	SE h^2	z h^2	p h^2	r^2	SE r^2	z r^2	p r^2	CV_A
PL	0.441	0.041	10.756	0.000	0.885	0.058	15.393	0.000	2.570
PW	0.357	0.047	7.596	0.000	0.675	0.049	13.787	0.000	2.260
FR	0.360	0.047	7.660	0.000	0.720	0.051	14.073	0.000	2.312
TL	0.367	0.042	8.738	0.000	0.870	0.054	16.243	0.000	2.462
TW	0.424	0.043	9.860	0.000	0.882	0.060	14.762	0.000	2.519
ST	0.027	0.131	0.206	0.837	0.391	0.136	2.867	0.004	0.453
AN	0.257	0.149	1.725	0.085	0.577	0.147	3.917	0.000	1.449
HT	0.172	0.212	0.811	0.417	- [a]	-	-	-	0.896
NF	0.000	0.176	0.000	1.000	-	-	-	-	0.010

[a] r^2 only available for traits with multiple measurements per plant.

PL: petal length; PW: petal width; FR: number of petal fringes; TL: corolla tube length; TW: corolla tube width; ST: stigma exsertion; AN: anther exsertion; HT: display height; NF: number of flowers.

Table 2. Phenotypic correlations (r_P, above the diagonal), additive genetic correlations (r_A, below the diagonal), with corresponding standard errors (in parentheses) among six floral traits of *Silene stellata*, calculated with data pooled across years using the multivariate animal model with year as a fixed effect. Significance levels were calculated for individual *z* scores based on a large sample standard normal distribution (*** P<0.001; ** P<0.01; *P<0.05; ns, not significant).

		Attraction			**Pollen transfer**		
		PL	PW	FR	TL	TW	AN
Attraction	PL	-	0.559 (0.047) ***	0.19 (0.056) ***	0.388 (0.052) ***	0.39 (0.049) ***	0.277 (0.049) ***
	PW	0.944 (0.260) ***	-	0.142 (0.054) **	0.208 (0.05) ***	0.527 (0.043) ***	0.353 (0.045) ***
	FR	0.310 (0.187) ns	0.324 (0.161) *	-	0.08 (0.055) ns	0.116 (0.054) *	0.141 (0.051) **
Pollen transfer	TL	0.466 (0.198) *	0.337 (0.145) *	0.178 (0.145) ns	-	-0.208 (0.053) ***	0.487 (0.039) ***
	TW	0.774 (0.257) **	0.663 (0.177) ***	0.283 (0.165) ns	-0.062 (0.138) ns	-	0.157 (0.05) **
	AN	0.626 (0.204) **	0.474 (3.12) **	0.296 (0.160) ns	0.972 (0.175) ***	0.163 (0.143) ns	-

PL: petal length; PW: petal width; FR: number of petal fringes; TL: corolla tube length; TW: corolla tube width; ST: stigma exsertion; AN: anther exsertion; HT: display height; NF: number of flowers.

Supplemental Table 1. Sample pseudo-diploid input file containing actual genotypes of 10 individuals at locus 3R with 20 alleles.

ID	Original tetraploid genotype				Transformed genotypes at 20 pseudo-diploid dominant loci																			
	Locus 3R (bp)				3R-142	3R-145	3R-148	3R-151	3R-154	3R-157	3R-160	3R-163	3R-166	3R-169	3R-172	3R-175	3R-178	3R-181	3R-184	3R-187	3R-190	3R-193	3R-196	3R-199
12001	148	160	169		0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
12011	145	148	154	169	0	1	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
12012	148	157	163		0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
12021	151	154	172	175	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
12031	145	148	169	181	0	1	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0
12041	148	154	187		0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
12042	148	154	157	160	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
12101	148	151	166	172	0	0	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
12111	148	157	175		0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
12121	148	151	163	169	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0

Supplemental Table 2. Heritabilities (h^2) and corresponding standard errors (SE), *z* scores (*z*), and significance from zero (*p)* of nine morphological traits of *Silene stellata* estimated for year 2012 and 2013 based on 11 microsatellite loci, using the univariate animal model. Also shown are the *z* scores and significance levels (*p)* for comparison of h^2 across years. Significance levels were calculated for individual *z* scores based on a large sample standard normal distribution.

Trait	**2012**				**2013**				**2012 vs. 2013**	
	h^2	SE	*z*	*p*	h^2	SE	*z*	*p*	*z*	*p*
PL	0.366	0.599	0.611	0.541	0.339	0.319	1.063	0.288	0.040	0.968
PW	0.530	0.284	1.866	0.062	0.538	0.185	2.908	0.004	-0.024	0.981
FR	0.344	0.495	0.695	0.487	0.363	0.350	1.037	0.300	-0.031	0.975
TL	0.351	0.427	0.822	0.411	0.310	0.289	1.073	0.283	0.080	0.937
TW	0.332	0.335	0.991	0.322	0.350	0.277	1.264	0.206	-0.041	0.967
ST	0.000	0.251	0.000	1.000	0.001	0.334	0.003	0.998	-0.002	0.998
AN	0.437	0.385	1.135	0.256	0.411	0.270	1.522	0.128	0.055	0.956
HT	0.052	0.389	0.134	0.894	0.044	0.399	0.110	0.912	0.014	0.989
NF	0.000	0.332	0.000	1.000	0.089	0.382	0.233	0.816	-0.176	0.860

PL: petal length; PW: petal width; FR: number of petal fringes; TL: corolla tube length; TW: corolla tube width; ST: stigma exsertion; AN: anther exsertion; HT: display height; NF: number of flowers.

Supplemental Table 3. Genetic correlations (r_A) and corresponding standard errors (SE), z scores and significance from zero (p) between six morphological traits of *Silene stellata* estimated for year 2012 and 2013, using the multivariate animal model. Also shown are the z scores and significance levels for comparisons of r_A across years. Significance levels were calculated for individual z scores based on a large sample standard normal distribution.

	2012					**2013**					**2013 vs. 2013**	
	r_A	SE	z	p		r_A	SE	z	p		z	p
PL vs. PW	0.933	0.762	1.224	0.221		0.915	0.417	2.194	0.028		0.021	0.983
PL vs. FR	0.406	0.483	0.841	0.401		0.354	0.314	1.127	0.260		0.090	0.928
PL vs. TL	0.340	0.448	0.759	0.448		0.350	0.354	0.989	0.323		-0.018	0.986
PL vs. TW	0.852	0.788	1.081	0.280		0.860	0.509	1.690	0.091		-0.009	0.993
PL vs. AN	0.525	0.451	1.164	0.244		0.551	0.357	1.543	0.123		-0.045	0.964
PW vs. FR	0.380	0.354	1.073	0.283		0.312	0.237	1.316	0.188		0.160	0.873
PW vs. TL	0.365	0.343	1.064	0.287		0.308	0.233	1.322	0.186		0.137	0.891
PW vs. TW	0.657	0.387	1.698	0.090		0.665	0.280	2.375	0.018		-0.017	0.987
PW vs. AN	0.417	0.254	1.642	0.101		0.410	0.210	1.952	0.051		0.021	0.983
FR vs. TL	0.158	0.293	0.539	0.590		0.186	0.257	0.724	0.469		-0.072	0.943
FR vs. TW	0.386	0.421	0.917	0.359		0.338	0.286	1.182	0.237		0.094	0.925
FR vs. AN	0.319	0.337	0.947	0.344		0.267	0.225	1.187	0.235		0.128	0.898
TL vs. TW	-0.091	0.266	-0.342	0.732		-0.069	0.229	-0.301	0.763		-0.063	0.950
TL vs. AN	0.916	0.606	1.512	0.131		0.949	0.486	1.953	0.051		-0.042	0.966
TW vs. AN	0.238	0.267	0.891	0.373		0.222	0.215	1.033	0.302		0.047	0.963

PL: petal length; PW: petal width; FR: number of petal fringes; TL: corolla tube length; TW: corolla tube width; AN: anther exsertion.

Figure 1. Association between heritabilities (h^2) estimated for year 2012 and 2013, using the univariate animal model. Pearson correlation coefficient r = 0.879, $p < 0.01$; Spearman rank correlation ρ= 0.854, $p < 0.01$. PL: petal length; PW: petal width; FR: number of petal fringes; TL: corolla tube length; TW: corolla tube width; ST: stigma exsertion; AN: anther exsertion; HT: display height; NF: number of flowers.

Figure 2. Association between genetic correlations (r_A) estimated for year 2012 and 2013 using the multivariate animal model. Pearson correlation coefficient r = 0.978, $p < 0.001$; Spearman rank correlation ρ = 0.975, $p < 0.001$. PL: petal length; PW: petal width; FR: number of petal fringes; TL: corolla tube length; TW: corolla tube width; ST: stigma exsertion; AN: anther exsertion; HT: display height; NF: number of flowers.

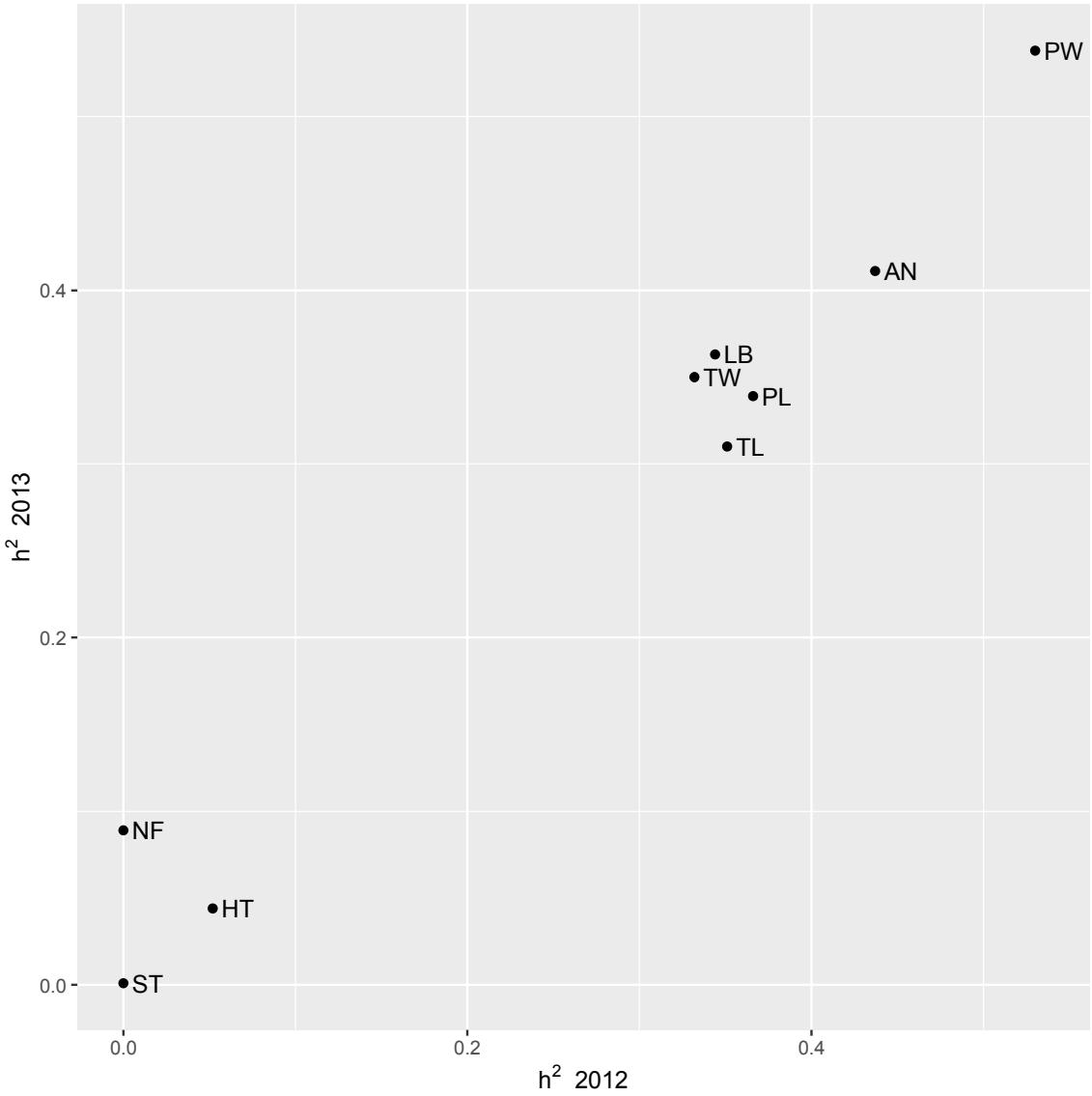


FIG. 1

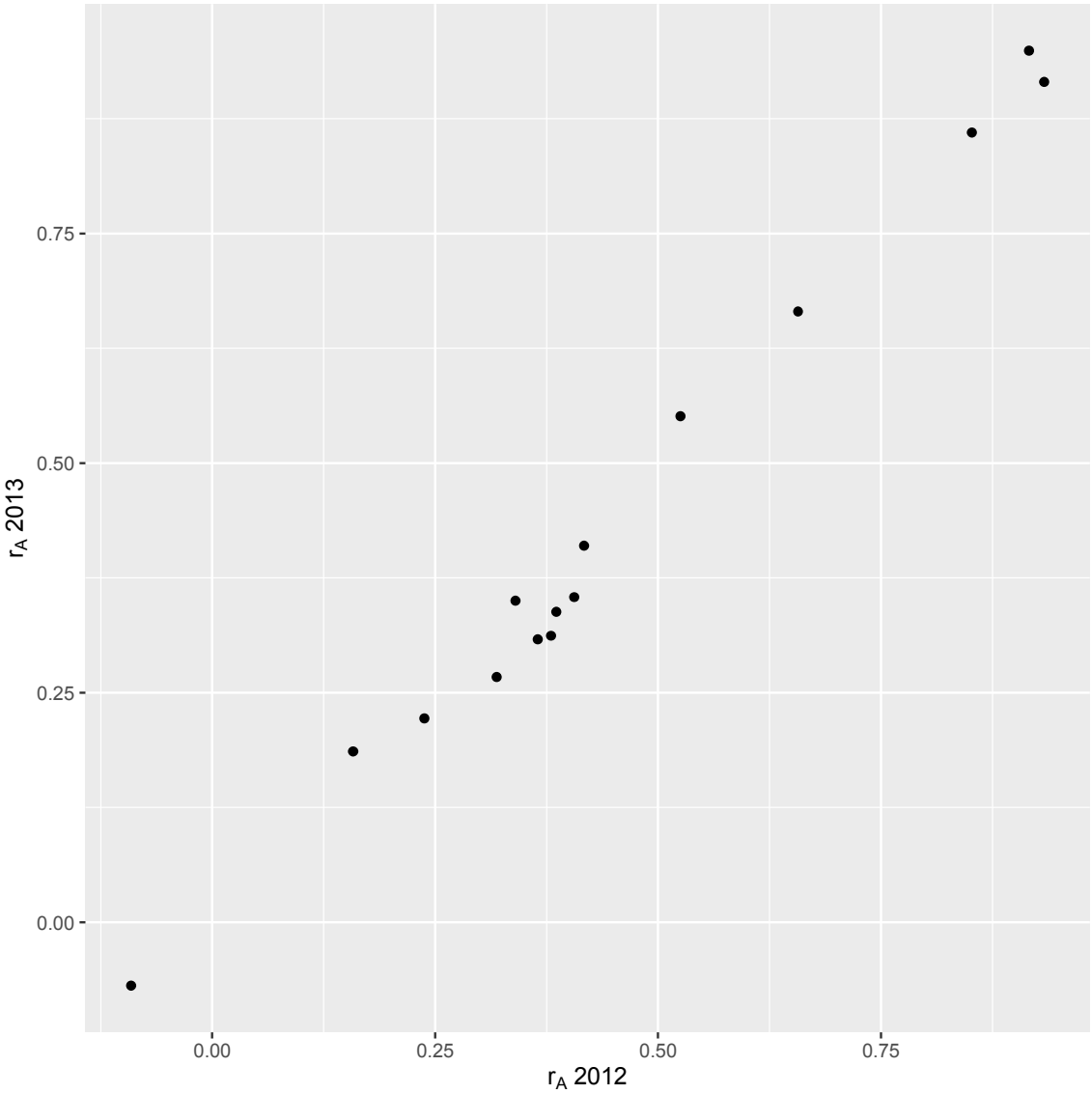


FIG. 2

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