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PRE-AND POST-ZYGOTIC ISOLATING BARRIERS IN *SILENE*

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Amanda Nicole Brothers

PRE- AND POST-ZYGOTIC ISOLATING BARRIERS IN *SILENE*

How boundaries are maintained between closely related species is one of the central questions in evolutionary biology. I addressed three questions regarding how species boundaries are maintained in closely related species of *Silene*. First I tested whether pollinator-mediated selection for particular floral traits shapes the phenotypes of *S. latifolia* and *S. diclinis*, thus contributing to pre-zygotic isolating barriers. These two species occur sympatrically and cross successfully in the greenhouse, although hybrids between the two have not been observed in the wild. I tested for both differential visitation and seed set using F2 hybrids to understand which traits may be important for pollinator-mediated selection. Floral visitors preferred short flowers during the day and tall flowers at night. Larger flowers were more likely to be predated at night. These results suggest that differential visitation by pollinators has shaped floral traits and that selection by pollinators may contribute to reproductive isolation between these two species in nature. Second, I investigated whether Haldane's Rule applies to plants. Haldane's rule states that in the F1 hybrid generation between two species, the heterogametic sex (e.g. XY) is more likely to be rare, absent, or sterile, thus providing a post-zygotic isolating mechanism. Haldane's rule has been observed in over 250 species of animals, but has not been documented in plants. *Silene latifolia*, *S. diclinis*, and *S. dioica*, are unique in that all three are dioecious with heteromorphic sex chromosomes. Males are heterogametic, although the sex chromosomes are relatively young. Male F1 hybrids exhibited rarity and sterility, extending Haldane's rule to plants. Finally, to further investigate what might cause chromosomal incompatibilities between *S. latifolia* and *S. diclinis* I used solid staining techniques to look at the sex chromosomes. Unlike *S. latifolia* or *S. dioica*, *S. diclinis* has neo-sex chromosomes. This is likely the result of a reciprocal translocation between the Y-chromosome and an autosome. Because *S. latifolia* and

S. diclinis successfully produce viable F1 hybrids, the neo-sex chromosomes of *S. diclinis* must be able to pair with species that do not have neo-sex chromosomes. Using solid staining techniques, we observed the arrangement of the X, Y, and neo-sex chromosomes in both pure species and hybrids. We found that the neo-sex chromosomes found in *S. diclinis* can be inherited across species and are not an absolute barrier to hybridization between *S. latifolia* and *S. diclinis* at the F1 generation. The results of these studies suggest that multiple pre- and post-zygotic barriers are important for maintaining species boundaries in dioecious *Silene*.

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CHAPTER 1

INTRODUCTION

Interest in understanding what promotes biological diversity and determining mechanisms preventing random mating, or panmixia, has fascinated scientists since Darwin. Vast progress in speciation research has greatly improved the ability of biologists to identify and quantify reproductive isolating barriers in a variety of different systems (as reviewed in Sobel et al. 2010). However, to understand the generality and variety of evolutionary processes across taxa, it is important for speciation research to continue to encompass a greater diversity of organisms and to identify both similarities and differences in how groups have diversified. Much of what is known about speciation mechanisms and processes comes from work in model systems with both genetic and genomic strategies for disentangling the complexities of gene flow between species (e.g. Rieseberg 2001, Orr 2001, Coyne and Orr 2004, Orr et al. 2007, Rieseberg and Willis 2007). The advantages of this type of research are numerous and have provided both a framework and a tool kit to tackle speciation questions. As additional work on a variety of species begins to incorporate both traditional and modern methods for assessing mechanisms that limit or allow gene flow between species, we can begin to fully understand the processes that generate and maintain the diversity of living organisms (Lowry et al. 2008).

In flowering plants there is an abundance of phenotypic and genetic divergence even among closely related species. Furthermore, the mechanisms by which plants diverge are numerous and generally characterized by multiple reproductive isolating barriers of various strengths between closely related species (Stebbins 1950, Lexer and Widmer 2008, Lowry et al. 2008, Kay and Sargent 2009). Both pre- and post-zygotic isolating barriers have contributed to species divergence and are important in plant speciation. Though few single studies have been able to address all of the potential barriers in a given species group, the field of speciation research has made great advances with regard to both the types of barriers being investigated and the variety of taxa included. **The work presented here focuses on three closely related plant species from the genus *Silene* and presents data from three studies examining both pre- and post-zygotic reproductive isolating barriers.**

One pre-zygotic barrier that has been characterized in flowering plants is a shift in floral visitors, which may exert divergent selection on floral traits (Schemske and Bradshaw 1999, Jones and Reithel 2001, Hoballah et al. 2007). Changes in pollinator visitation have been shown to correspond with changes in floral traits such as color, morphology, and odor (reviewed in Kay and Sargent 2010). Furthermore, changes in color and morphology resulting in pollinator shifts, have been shown to correspond to changes at a QTL or even a single locus (Bradshaw and Schemske 2003, Hobollah et al. 2007, Bouck et al. 2007). However, questions regarding the general importance of pollinators for divergent selection on floral traits have been raised (Waser et al. 1996, Ollerton 1996). Field surveys reveal that the majority of plants are visited by more than one pollinator (Robertson 1928). This challenges the idea of a one-to-one relationship between plants and their pollinators as originally proposed by Stebbins' most effective pollinator principle (1950). Additionally, the ephemeral nature of plants and pollinators, both spatially and temporally, requires that most groups generalize. This discrepancy can be reconciled by grouping species of insects that exert similar selection pressures into functional groups (Fenster 2004). Consequently, functional groups that exert similar selection pressures are thought to have shaped floral phenotypes. Similarly, opposing selection on divergent floral traits by different functional groups of pollinators may contribute to speciation. However, some studies of pollinator-mediated selection on floral traits have found that pollinator selection is not sufficiently strong to prevent gene flow (Wesselingh and Arnold 2000, Gegear and Burns 2007, Cooley et al. 2008). Thus, while pollinators may select which flowers to visit based on traits, their lack of fidelity among species is not generally sufficient to halt gene flow completely. However, there are many other isolating barriers in plants that can prevent gene flow in sympatric populations. Key among them are flowering time (Martin et al. 2007), con-specific pollen precedence (Montgomery et al. 2009), seed abortion (Chari and Wilson 2000), and Dobzhansky-Muller incompatibilities (DMI), all of which limit gene flow between sympatric plant species.

One pattern that has been largely attributed to DMI's and that has been found throughout the animal kingdom is Haldane's rule, which states that when two species or races are crossed, the heterogametic sex is more likely to be rare, absent, or sterile (Haldane 1928), thus discouraging or preventing gene flow. The pattern has been found in over 244 species of animals, with both XY and ZW systems conforming to the rule. To date, studies of Haldane's rule have been restricted to the animal kingdom, but approximately seven to ten percent of plant species are dioecious (separate males and females), and sex in some species is determined by sex chromosomes. Investigating Haldane's rule and the mechanisms underlying genetic incompatibilities in dioecious plants provides a novel opportunity to compare speciation processes between plants and animals.

Study Species

Silene latifolia, *S. diclinis*, and *S. dioica* are three closely related, dioecious plant species. All three are native to Europe and in each species, sex is determined by heteromorphic sex chromosomes. *Silene latifolia* occurs in sympatry with both *S. dioica* and *S. diclinis*, although the latter two species do not co-occur. Both *S. latifolia* and *S. dioica* are widely distributed throughout Europe (as well as the United States, where they have naturalized). In contrast, *Silene diclinis* is an endangered, endemic species restricted to a small (9 x 20 km) area of southeastern Spain (Prentice and Andersson 1977, Montesinos et al. 2006). Although hybrids between *S. latifolia* and *S. dioica* occur in the field, there is no evidence for genome-wide admixture (Minder 2007, Karrenberg and Favre 2008). Phenotypically detectable hybrids between *S. latifolia* and *S. diclinis* have not been observed in the wild (Prentice 1976, Brothers, pers. obs.).

Among dioecious plants, the degree to which the sex chromosomes have differentiated varies widely. In *Silene*, the Y-sex chromosome is estimated to be between five- to ten-million years old (my) (Nicolas et al. 2005). In contrast, mammalian sex chromosomes are estimated to be on the order of 200 my (Lahn and Page 1999, Nicolas et al. 2005). *Silene latifolia*, *Silene dioica*, and *Silene diclinis* are thought to have shared a single dioecious ancestor (Desfeux 1996), with *S. latifolia* and *S. dioica* being more closely related to one another than either is to *S. diclinis* (B. Oxelman, Pers. Comm). The research presented here examines some of the potential barriers to gene flow enhancing reproductive isolation between *S. latifolia*, *S. diclinis*, and *S. dioica*.

Overview of chapters

Chapter 2 explores pollinator-mediated selection as a pre-zygotic isolating barrier between *S. latifolia* and *S. diclinis,* using field observations of an independently segregating population of F2 hybrids. The two species have dramatically different floral and vegetative characteristics, and previous work in other systems has suggested that pollinators may be important for preventing pollen transmission between closely related species in sympatry (reviewed in Kay and Sargent 2009). This work was done in collaboration with Jonathan Atwell. In Chapter 3, I present a test for Haldane's rule in plants, presenting data on the levels of sterility and inviability found in F1 hybrid offspring between the three species in a greenhouse experiment. To date, Haldane's rule has been observed in over 244 animal species. However, the occurrence of Haldane's rule has not been documented in plants. This research was conducted in collaboration with Dr. Lynda Delph. In Chapter 4, I present research examining the sex chromosomes of both *S. latifolia* and *S. diclinis* and their hybrids using fluorescent in situ hybridization (FISH). One of the unique features of *S. diclinis* is the presence of neo-sex chromosomes (Howell et al 2009). The purpose of this chapter was to identify the nature of sex chromosome movement in hybrids between species with and without neo-sex chromosomes. Chapter 4 was done in collaboration with Victoria Jideonwo and Lynda Delph.

CHAPTER SUMMARIES

CHAPTER 2:

It has been proposed that pre-zygotic barriers between closely related species in sympatry are likely to be an important first step in the speciation process, and may also serve to reinforce or maintain isolation. While *S. latifolia* is found throughout Eastern Europe, *S. diclinis* is an endangered endemic species found only in the southeastern region of Spain. Despite their close proximity (in some places they occur within a few kilometers of each other), phenotypically detectable hybrids have not been observed in the field, although crosses in the greenhouse produce viable hybrids. We therefore hypothesized that pollinator isolation may play an important role in preventing gene flow between these two species. The two species vary dramatically in floral traits. *Silene latifolia* is tall, with white flowers, and is primarily pollinated by the night flying moth, *Hadena bicruris*, which is a nursery pollinator. In contrast, *S. diclinis* is low growing, with pink flowers, and pollinated by generalist bees and flies. In order to separate individual traits from their associated floral syndromes, we generated a population of F2 hybrids that appear to segregate independently for the traits of interest. The F2 arrays were placed in the field near native plant populations of both *S. latifolia* and *S. diclinis* and observations of floral visitors were recorded. Additionally, we conducted exclusion experiments in which pollinators were excluded either during the day or at night to determine the overall fitness associated with particular traits during the day or at night. In both experiments, we looked at pollinator preferences for seven floral traits as well as interactions among the traits. We found that flower height influenced pollinator visitation. We also found that increased flower size improved seed set, but also increased predation rates. Flower color did not significantly increased pollinator visitation, seed set, or predation. We conclude that pollinators may play a role in pre-zygotic isolation in this system, however, pollinator-mediated selection does not act as an absolute reproductive isolating barrier in these two species.

CHAPTER 3: HALDANE'S RULE IS EXTENDED TO PLANTS WITH SEX CHROMOSOMES

Haldane's rule states that in hybrids between species, the heterogametic sex is more likely to be sterile or absent (Haldane 1922). Remarkably, this pattern has been observed in well over 200 species of animals. However, the applicability of Haldane's rule to species outside of the animal kingdom is not well documented. One of the unique aspects of *Silene latifolia*, *Silene dioica*, and *Silene diclinis* is that they have heteromorphic sex chromosomes, and that the sex chromosomes (at least those of *S. latifolia*) are relatively well studied. Moreover, the sex chromosomes found in *Silene* are much younger (5-10my) compared to those of animals (~200my), providing an opportunity to evaluate whether Haldane's rule applies to more recently derived chromosomes. In *Silene*, the Y chromosome is much larger than the X chromosome meaning that it has likely undergone less selection than the Y chromosome of mammals. To test for Haldane's rule in *Silene,* we performed reciprocal crosses between *S. latifolia, S. diclinis*, and *S. dioica* in order to investigate whether plants with sex chromosomes exhibit Haldane's rule.

We found evidence supporting Haldane's rule for both inviability and sterility in males relative to females. Our findings demonstrate that both inviability and sterility may occur relatively early in the differentiation of sex chromosomes and that Haldane's rule extends beyond the animal kingdom. These findings may have implications for understanding the early evolution of sex chromosomes. Furthermore, the diversity of plants and their breeding systems provides a new avenue of research in which to test theories about the causes of Haldane's rule.

CHAPTER 4: THE FATE OF NEO-SEX CHROMOSOMES IN HYBRIDS OF DIOECIOUS SILENE WITH HETEROMORPHIC SEX CHROMOSOMES

Dioecy (separate males and females) is found in a relatively small number of plants estimated to make up only about 5-10% of plant taxa. Of those, an even smaller number have heteromorphic sex chromosomes. However, of plants with heteromorphic sex chromosomes, those found in *Silene latifolia* are well studied. While the closely related sister species, *S. diclinis,* has received less attention, it was recently reported that *S. diclinis* has neo-sex chromosomes as a result of a translocation between the Y chromosome and an autosome (Howell et al. 2009). Furthermore, Howell et al. (2009) conclude that hybrids between *S. latifolia* and *S. diclinis* are inviable and they attribute this to the neo-sex chromosomes of *S. diclinis*. We know that hybrids between these two species are both viable and capable of producing offspring. Thus, while it is somewhat surprising that we are so readily able to produce hybrids between *S. diclinis* and *S. latifolia,* we have evidence that these species are capable of interbreeding. Consequently, we examined how the neo-sex chromosomes of *S. diclinis* pair with *S. latifolia*, which does not have neo-sex chromosomes, and we identify the Y chromosomes, X chromosome, and Y2 chromosome in hybrids. We found that in hybrids between *S. latifolia* and *S. diclinis,* in which *S. diclinis* is the paternal parent, the Y chromosome and Y2 or neo-Y chromosome, are both inherited from the father. We also explored some fluorescent *in Situ* hybridization (FISH) techniques for identifying specific regions of the X chromosome, Y chromosome, and neo-Y chromosome in hybrids of these two species.

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CHAPTER 2

THE ROLE OF POLLINATOR-MEDIATED SELECTION IN THE DIVERGENCE OF FLORAL TRAITS BETWEEN TWO CLOSELY RELATED PLANT SPECIES

Amanda N. Brothers and Jonathan W. Atwell

ABSTRACT

Floral traits are important for pollinator attraction and efficient pollen transfer within species. Selection on floral traits by pollinators is therefore expected to play an important role in shaping floral phenotypes and maintaining species boundaries. However, some traits may be more important for attracting pollinators than others. Evaluating individual traits and suites of traits independent of their expected pollination syndrome can provide insight into the mechanisms that generate and maintain floral diversity. We created F2 hybrids between two sympatrically distributed sister species of *Silene* to segregate floral characteristics from their associated pollination syndromes. We observed natural pollinators and experimentally excluded pollinators from arrays of plants in order to address whether seven individual traits, or combinations of traits, influenced pollinator visitation, seed set, or predation. *Silene latifolia* flowers at night and was primarily pollinated by the nursery pollinator, *Hadena bicruris*. *Silene diclinis* flowers during the day and was pollinated by bees and flies. We found evidence for divergent selection by pollinators on ecological traits that likely have contributed to divergence in the floral phenotypes of these two species. Flower height best predicted visitation during both the day and night but in opposite directions, suggesting disruptive selection by pollinators. During the day, bees and flies visited short plants more, and at night, moths preferred tall plants. Seed set was higher in plants with large flowers during the day however, moths predated large flowers at night, suggesting that balancing selection may act against large flowers in *S. diclinis* where predation by moths may be disadvantageous. Interestingly, floral color was not associated with visitation rates, seed set, or predation in our study, despite its importance for pollinator selection in other systems. We conclude that floral phenotypes are the result of selection on multiple traits by a variety of

forces. These findings highlight the complex nature of how floral characteristics are shaped and demonstrate that floral traits may be important for maintaining species boundaries.

INTRODUCTION

One of the most fascinating aspects of plant evolutionary biology is the astounding range of floral forms, and biologists have long sought to understand the mechanisms that generate and maintain floral diversity. The diversification of flowering plants has been linked to competition for animal pollinators, and divergence among plants and pollinators can contribute to isolation between species (Grant 1994). However, some floral traits may be more important than others to pollinators. Pollination syndromes, or suites of floral traits such as color, shape, and size, are often highly specialized between specific plant species and their preferred pollinators. Pollination syndromes are important for insect attraction and help to insure the most efficient transfer of pollen within species, and this is predicted to lead to strong selection on floral traits important as cues to insect pollinators. Understanding how floral traits contribute to pollinator attraction and predator avoidance requires examining individual traits independent of their associated pollination syndrome. While selection on traits important for pollinator attraction and gamete dispersal should be strong, it is important to distinguish between traits that are important for pollinator attraction versus those that may repel other visitors. Furthermore, correlations among traits can cause cascading effects on traits that may be less important (Bhattacharyay and Drossel, 2005).

Groups of floral traits are frequently associated with a specific set of animal pollinators, and generalized functional groups of pollinators and their corresponding floral types have been characterized (Fenster 2004). Examples of pollination syndromes include those associated with hummingbirds (e.g., red flowers, long corollas, and large nectar rewards), moths (e.g., white flowers, large petals, and strong odors) and bees (e.g., bright colors, sweet-smelling flowers). Although much research has focused on the one-to-one relationships exhibited by plants and pollinators such as those exhibited by figs and fig wasps (Anstett, 2001; Westerbergh and Westerbergh, 2001; Grison-Pige, Bessiere, and Hossaert-McKey, 2002) or yuccas and yucca moths (Huth and Pellmyr, 2000; Svensson et al., 2005), the majority of plants are pollinated by multiple species which challenges the idea of specialization (Robertson 1928). This means that plants must be able to attract and retain multiple pollinators in order to disperse sufficient pollen. The challenge presented by multiple pollinators to explaining the influence of pollinators on floral traits can be resolved by assuming that classes of pollinators exert similar selection pressures (Waser 1996, Fenster 2004). Similarly, differing preferences for particular floral traits by groups of pollinators may be important for preventing gene flow among closely related species.

Floral traits such as flower color, nectar volume, flower shape, and flower orientation are known to influence pollinator attraction and fidelity (Faegri and van der Pijl 1979, Fulton and Hodges 1999, Melendez-Ackerman et al 1997, Schemske & Bradshaw 1999, Fenster 2004, Gegear 2005, Wolfe and Sowell 2006). Traits important for plant fitness should be under strong selection and functionally related traits should have evolved together to reach fitness optima (Arnold 1992). Furthermore, selection by pollinators on floral traits that are genetically correlated can underlie phenotypic integration, which may constrain how floral traits respond to selection (Frey 2007, Perez et al. 2007). On the other hand, previous studies have shown that pollinator-mediated selection can act on individual traits separately (Melendez-Ackerman et al 1997) or on combinations of traits. Therefore, it is important to examine multiple individual traits and combinations of traits within a study for a comprehensive view of pollinator-mediated selection. However, studies attempting to simultaneously consider the importance of multiple floral traits for pollinator attraction and plant fitness are often limited in their conclusions because of constraints imposed by correlations among traits.

Flower color is known to be important for pollinator attraction (Robertson and Wyatt 1990, Schemske and Bradshaw 1999, Frey 2004). Simulations of pollinator preference for color have indicated that even slight preferences by one pollinator for a specific color are sufficient for isolation between species (Gegear and Burns 2007). In F2 hybrids of *Mimulus,* bees were shown to prefer pink flowers over red or orange flowers regardless of flower nectar volume; however, hummingbirds had equal visitation rates regardless of color, but visited plants with higher nectar volume more frequently (Schemske and Bradshaw 1999). This suggests that color may be important for attracting some pollinators, but may not be important to other groups of pollinators.

Flower shape and size can provide cues to pollinators for locating and landing on flowers (Fulton and Hodges 1999, Gomez et al. 2006). Shifts in floral shape and size in response to pollinator-mediated selection have been demonstrated for several floral traits including corolla length, corolla diameter, and flower area (Galen 1989, Schemske and Bradshaw 1999). Species in which female pollinators lay their eggs inside the calyces of flowers may also use flower size as an indication of food availability for offspring. Flower height has also been shown to influence pollination success (Cariveau et al. 2004, Medrano et al 2006) and has been shown to vary in response to different pollinator species (Galen 1989).

While the underlying genetic mechanisms of floral transitions are just beginning to be identified, a few studies have been able to demonstrate that just one or a few genes of major effect can cause rapid shifts in floral morphology and consequently, pollinator visitations (Bradshaw and Schemske 2003, Hobollah et al. 2007) suggesting that pollinator-mediated selection plays an important role in shaping floral traits. One method for visualizing how pollination syndromes are shaped is to observe pollinators visiting plants whose traits have been disassociated from their respective pollination syndromes. Here we examine pollinator visitation rates, seed set, and predation for multiple individual traits and combinations of traits in female F2 hybrids that have been disassociated from their respective pollination syndromes.

The goal of this research was to determine how floral traits influence pollinator-mediated selection in *Silene latifolia* and *Silene diclinis*. This study focused on which traits are attractive to floral visitors. Because visitation and pollination are two different phenomena we chose to address the question of whether particular traits are important by using two different approaches. We examined whether individual traits or suites of traits influenced insect visitation to artificially generated F2 hybrids by observing floral visitors within native populations. This approach was intended to isolate individual traits from their usual pollination syndrome to observe whether there are particular traits that can be used to predict visitation by pollinators. We also investigated differential selection by using seed set and predation damage as measures of fitness to determine which traits best predicted pollination or predation.

MATERIALS AND METHODS

Study species

Silene latifolia and *S. diclinis* are two closely related species that co-occur in the southeastern region of Spain. Both species are dioecious (separate males and females) and pollination requires visitation by a single pollinator to both a male and a female plant. Sex is determined by sex chromosomes and males are the heteromorphic sex.

Silene latifolia is broadly distributed throughout Europe and has also become established in North America. *Silene latifolia* is found in a wide range of habitat conditions and is considered a weedy species. The pollination syndrome of *S. latifolia* is characterized by tall, erect flowering stems with large white flowers that have a musky scent. The ovaries of *S. latifolia* produce on average 383±11.15 ovules (n = 103) (Brothers and Delph unpublished data). Within its native range, *S. latifolia* is most frequently pollinated by the nocturnal moth, *Hadena bicruris*, which is a nursery pollinator (Young 2002, Wright and Meagher 2003). The flowers of *S. latifolia* are also visited by diurnal visitors who are less efficient at pollen transfer (Young 2002). The flowers of *S. latifolia* are crepuscular and will close in the late morning on hot days and re-open again in the evening, though they may remain open throughout the day on cool or cloudy days.

Silene diclinis is an endangered endemic species found only in a small region (18km x 9 km) of Valencia, Spain where there are efforts to protect it from encroaching habitat loss (Montesinos et al. 2006). *S. diclinis* is pollinated by generalist bees and flies during the day. The restricted range of *Silene diclinis* has been attributed to limited seed dispersal, which is carried out by granivorous ants (Montesinos et al. 2006). *Silene latifolia* is found throughout the range of *S. diclinis* with some populations occurring in sympatry (less than 400m from each other) and well within the traveling distance of *H. bicruris*. Despite their close geographic overlap within the Valencia region of Spain, phenotypically detectable hybrids between the two species have not been observed in the field (Prentice 1976, Brothers pers. Obs., 2009). *Silene diclinis* has short, prostrate stems and sweet smelling, brightly colored, pink flowers. A typical ovary has 70±1.5 ovules (n=101) (Brothers and Delph unpublished data), thus individual fruits produce far fewer seeds than *S. latifolia*.

Study species and generation of F2 hybrids

Hybrid crosses were performed in the greenhouse between *S. latifolia* and *S. diclinis* to yield F1 hybrids with intermediate phenotypes. Crosses between F1 hybrids of all were performed to generate F2 hybrid's with a wide range of trait combinations. All crosses were done under standard greenhouse conditions at Indiana University and seeds were mailed to Spain where they were grown. The *S. latifolia* parents used in this study were from a population in Alencon, France and the *S. diclinis* parents were from Xativa, Spain.

Study site and measurements

Hybrid F2 plants used in the field experiment were grown in 4-inch pots in greenhouses in two locations. One set was grown in Valencia, Spain where they were maintained by the Centro de Investigaciones sobre Desertificación (CIDE). A second set of F2 hybrids were grown in a private greenhouse near Coimbra, Portugal. Once basal rosettes were established, but prior to flowering, plants were transported in their pots to Xativa, Spain where they were maintained from April to June of 2009.

Plants were kept outside under several layers of bridal veil netting to prevent pollinators from visiting between pollinator observation trials. Upon flowering, measurements were recorded for seven floral and vegetative traits. Floral traits were measured for the first three flowers per plant and included four flower size measurements: calyx width (cw), calyx length (cl), petal-limb length (pll) and petal cleft (clft). Flower size measurements were taken using digital calipers and averaged over the three flowers for each individual. To measure flower color, each plant was scored based on visual observations as white, light pink, pink, or bright pink. Additionally, petals were collected from each plant and photos were taken in groups of 24 by arranging petals on a board with black material. A ColorChecker chart (GretagMacBeth) was placed in the center of each board to serve as a color control (Supplemental Fig. 1). The ColorChecker chart is designed to mimic colors found in nature and thus has appropriate control colors. The photographs of each board were taken under similar light conditions and then analyzed in Adobe Photoshop using the inCamera 4.5 filter plug-in (methods described in detail in Bergman and Beehner 2008). First flower height and stem angle were also measured after the first flower opened. Only female plants were used in arrays in the field to prevent gene flow into native *S. diclinis* populations. There were a total of 463 female plants used in the study including pure species and hybrids and size, color, and height traits assorted independently in the F2 generation (Fig 2). The female plants were divided into twelve groups based on the full-factorial combination of the following three floral characteristics: petal-limb length (big or small), first flower height (tall or short), and color (white, light pink, or pink). Average size was calculated for petal-limb length and flower height. Plants with average petal-limb length measurements from across three flowers that were above the mean of the population were considered big (>7.8mm), and those that fell below were small. Similarly, plants with stems taller than average were considered tall (>19.3 cm), and those below the average were short. Plants were set out in the field in arrays of twelve individuals, one from each trait group, such that each of the twelve trait combinations was represented once in each array.

Pollinator observations

Pollinator observations were conducted during May and June of 2009, which is peak flowering season for *S. diclinis* and *S. latifolia* in this region. All observations were conducted in the Valencia region of Spain near Xativa, where there are several established populations of *S. diclinis*. *Silene latifolia* is also found throughout the area. For each observation a single plant from each group (i.e. big, tall, white) was randomly chosen and placed in an array comprised of twelve individuals representing a diverse array of traits. Pure species *S. latifolia* and *S. diclinis* were also used to represent the two extreme groups for some arrays. Arrays were arranged randomly and placed either in or adjacent to several natural populations of *S. diclinis* and *S. latifolia* and observed for one-hour intervals. Arrays were generally placed in or near large patches of *S. diclinis* during the day and in or near *S. latifolia* at night in order to observe the most pollinators in a single trial, as overall, pollinators to these species are not abundant. A single observer could watch up to two arrays per hour in a given area of *S. diclinis* or *S. latifolia* unless an unusual number of pollinators were present, which happened rarely in the course of the study. Prior to each observation period, the number of flowers on each plant, as well as the position of the plant in the array, were recorded. Pollinator observations were conducted during peak activity in both the late morning (9:00 am – 12:00pm) and evening (8:00pm – 11:00 pm) when visitation for each set of pollinators was at its highest. Day visitors were considered those observed during morning observations. Night visitors were those observed in the late evening. With few exceptions, most visits were considered an opportunity for pollen transfer as the stigmas of *S. latifolia* and *S. diclinis* are exerted and often extend onto the petal surface such that even a brief visit could potentially result in pollen transfer.

Analyses of pollinator observations

All data were analyzed as continuous variables; finite variables were only used for initial array selection. Because some plants were never visited in our arrays (zero data), a zero-inflated negative binomial generalized linear mixed model (GLMM) was used to analyze the influence of particular traits on visitation rates. This type of model allowed us to adjust the degrees of freedom because many plants were used in multiple arrays, and allowed us to consider the random effect of individual plant identity including the possible effects of traits that were not measured (e.g. scent). The total number of visits a plant received during an array observation was used as the dependent variable. Independent variables initially included color score, flower height, angle, calyx width, calyx length, petal-limb length, petal cleft, and the number of flowers. To simplify the model and account for correlations among traits, flower height and angle were combined into a single significant principal component to describe the overall height of flowers found on a plant (Table 1a). Similarly, calyx width, calyx length, petal limb length, and petal cleft were combined into a single significant principal component to describe the flower-size factor (Table 1b). Color measurements, which were composed of three measurements corresponding to red, green, and blue reflectance values, were also combined into a color principal component (Fig. 1/Table 1c).

Pollinator exclusions and seed set

Two sets of four arrays (as described above) totaling 48 plants per treatment (day vs. night) were assembled. An exclusion structure, made by draping bridal veil netting over a wooden frame, was built over each treatment set. All flowers were marked by placing a small piece of flexible wire around the pedicel. The day treatment was open to pollinators from 7:00 am until 7:00pm. The night treatment was open to pollinators from 7:00 pm until 7:00 am with the netting being transferred between treatments twice daily (7:00 am and 7:00 pm).

All new flowers were marked with wire each day. The experiment ran for fourteen days. At the end of fourteen days, both treatments were covered and left for five days so that all visited flowers had time to set fruit. After five days, the total number of flowers (number of wires), number of set fruits, and number of predated fruits were counted. Any fruit with seeds, regardless of whether it was predated, was collected and preserved in 90% ethanol. Collected fruits were brought back to the laboratory at Indiana University where the number of developing seeds and the number of unfertilized ovules were counted under a dissecting scope. If collected fruits contained larvae, they were considered predated, but developing seeds and ovules were still counted. Predation was attributed to larvae of *Hadena* moths. Fruits were considered predated if larvae or frass were found, or if evidence of larvae having previously inhabiting the fruit was evident.

ANOVA's were used to test the effect of day or night pollination. Average seed set per plant and predation were used as independent variables with color, size, and height factors as the covariates. Number of flowers was removed from the analyses because it did not significantly improve the models.

In considering the fitness component of seed set we also allowed for the possibility that a plant's reproductive potential may be limited by the number of ovules present, thus we computed seed set as a proportion of total ovules present. However, these proportions were always low (0.18±0.015), indicating that plants are probably pollen limited so we did not include these proportional measures in our analysis and reported only average number of seed set per fruit per plant. Furthermore, we found that ovule number was positively correlated with calyx width and hence this value is considered in our analysis as part of the size factor (by proxy) in so far that size factor is included in the model.

RESULTS

Pollinator visitation

A total of 100 hours of day watches and 100 hours of night watches were conducted on 200 arrays, 463 plants, and 11,867 flowers. A total of 1,148 visitors were observed. For each visit, the type of insect, duration of visit, and behavior were recorded. There were dramatic differences in frequency and abundance of pollinators observed during the day and at night. Day visitors included solitary bees (n = 148), bee-flies (n = 141), butterflies (n = 72), small beetles (n = 27), other bees (n = 25), and flies (n = 4). The majority of night pollinators were the nursery pollinator *Hadena bicruris* (n = 728) with a small number of sphinx moths (n = 34). Flower size, flower color, and flower height all assorted independently (Fig. 2) and hybrids encompassed the range of variation found within both parental species as well as exhibiting intermediate phenotypes (Fig 2). Variables included in the final GLMM were the height principle component (PC), the number of flowers for each plant, and the size PC. The interaction between day and night was also included for the height factor and the number of flowers, but the interaction term was not significant with regard to the size PC or the color PC, and the color PC was removed from the model entirely because it did not significantly improve the model.

Visitation increased with respect to height for both day and night pollinators in the direction expected by their associated pollination syndrome (Fig 3a). Pollinator visitation during the day was significantly higher on plants with flowers closer to the ground either as a consequence of short stems or a low stem angle ($Z = -2.71$; $P < 0.001$; Table 2, Fig 3a). Night visitation increased in plants with tall flowering stems ($Z = 2.68$; $P < 0.001$; Table 2, Fig 3a).

Both during the day and at night visitation was higher on plants with large flowers ($Z = 3.14$; $P < 0.001$; Table 2, Fig 3b) and plants with many flowers ($Z = 5.20$; $P < 0.001$; Table 2, Fig 3c).

Pollinator exclusions and seed set

The day and night treatment arrays did not produce significantly different numbers of flowers ($t = -0.538$, $p = 0.593$; Table 3), fruits ($t = -0.348$, $p = 0.729$; Table 3), or ovules per fruit ($t = -0.252$, $p = 0.802$; Table 3), thus the two treatments were considered to offer equivalent arrays over the course of the study. Predation on fruits by moths was significantly higher at night ($t = -5.424$, $p < 0.001$; Table 4) as was the number of larvae found in fruits at the end of the study ($t = -4.0$, $p < 0.001$; Table 4). Only one *Hadena* larvae was found in the day treatment, demonstrating that the nighttime exclosures were effective for excluding night-visiting pollinators. Seed set was higher in plants left open to pollinators at night ($t = -2.545$, $p = 0.014$; Table 4). The principal components for height, size, and color (as described previously) were also used to determine the importance of particular traits on fruit set, seed set, and predation. Neither height of the plants nor the color of the petals increased seed set, regardless of the time of day (Table 4). Size of the flowers did not significantly improve seed set at night, but larger flowers improved seed set during the day ($F = 5.702$, $p = 0.021$; Table 4, Fig 4a). An ANOVA was used to calculate the effects of predation. Because predation during the day was negligible (1 fruit) data are not presented. Large flowers at night were significantly more likely to be predated ($F = 5.543$, $p = 0.023$, Fig 4b) and white flowers showed a trend towards increased predation ($F = 3.284$, $p = 0.077$). Height was not a significant factor for predation ($F = 0.139$, $p = 0.711$).

DISCUSSION

We found that different floral traits influence different aspects of pollinator visitation, seed set, and predation in F2 hybrids of *Silene*. By assessing traits independently of their associated pollination syndrome, we evaluated how pollinators use individual floral cues across several traits to identify desirable flowers. Increased visitation corresponded with short plants during the day and tall plants at night (Fig 3a). Predation on plants was higher in individuals with large flowers regardless of flower height (Fig 4b). In contrast, visitation, seed set, and predation did not vary with flower color, which suggests that only some traits that make up a particular pollination syndrome may be associated with increased fitness. We conclude that selection is influenced by both pollination and predation and that investigating individual floral traits may improve how pollination syndromes are interpreted.

Evaluation of floral traits

Flower color, flower size, and flower height assort independently of each other in F2 hybrids of *S. latifolia* and *S. diclinis* (Fig 2). However, we found that correlations among flower size traits including calyx width, calyx length, petal limb length, and petal cleft were still present in the F2 generation. This finding suggests that flower size may be under the control of few genes or closely linked genes that do not segregate independently or that flower traits are constrained by developmental pathways. The height of F2 plants was strongly correlated with the stem angle because a lower angle resulted in flowers being closer to the ground. Consequently, flower size and flower height were analyzed as principal components that accounted for the variation among the correlated traits.

We predicted that diurnal pollinators would prefer either some or all of the traits associated with *S. diclinis.* For example, we expected bees and flies to prefer short-stemmed plants with small, pink flowers. In contrast, we expected that nocturnal pollinators would select for traits associated with the *S. latifolia* phenotype, or tall plants with large, white flowers. The results indicate that pollinator visitation is influenced by flower height and flower size, as well as the number of flowers (Fig 3). There was evidence for differential selection by both diurnal and nocturnal pollinators on flower height (Fig 3). Diurnal pollinators visited short plants more frequently then tall plants but preferred large flowers to small flowers and showed no preference for color. Previous studies have shown that bees prefer short plants, which can optimize foraging and conserve energy (Johnston 1991, Gumbert and Kunze 1999). Nocturnal pollinators visited tall plants more frequently and also preferred larger flowers. The most common nocturnal visitor to *S. latifolia* is *Hadena bicruris,* which lays its larvae in the fruit of *S. latifolia* (Young 2002). While large flowers are thought to be more attractive to pollinators and it is also likely that *Hadena* select for large flowers to provide more resources for offspring. Plants with more flowers received more visits. Flower number has been shown to vary among populations of *S. latifolia* (Wright and Meagher 2004) and is also highly dimorphic between the sexes. Males make more flowers relative to females and flower number is correlated with other floral and vegetative traits between the sexes (Delph et al. 2002, 2004, 2005). This means there is a possibility for selection on floral traits between the sexes to vary. However, male and female *Hadena* moths have been shown to visit *S. latifolia* male and female plants at the same rate (Labouche and Bernasconi 2010). Thus, while we could not assess male floral traits in the field without risking gene flow from hybrids into the endangered species, *S. diclinis*, at least with respect to the behavior of *Hadena* moths, results should apply to both male and female flowers, even though this study is limited to considering pollinator-mediated selection on floral traits to females.

It is somewhat surprising that color did not significantly improve pollinator visitation or seed set in F2 hybrids, as transitions in flower color have been demonstrated to influence pollinator visitation in numerous species (Campbell et al. 1997, Melendez-Ackerman et al. 1997, Melendez-Ackerman and Campbell 1998, Schemske and Bradsahw 1999, Bradshaw and Schemske 2003). However, it has also been suggested that flower-color transitions are not necessarily caused by selection via pollinators. Ecological non-pollinator selective forces such as genetic drift or pleiotropy are potential explanations for shifts in flower color (Irwin and Strauss 2005, Rausher 2008). For example, the enzymes required for anthocyanin synthesis are also necessary for plants to manufacture other flavonoids which are important for a number of floral and vegetative traits in addition to color (Rausher 2008). Consequently, selection for other physiological and ecological traits with pleiotropic effects may influence flower color (Strauss and Whittall 2006, Rausher 2008) in *Silene*.

Interestingly, for the pure species individuals included in the study, moths were never observed on *S. diclinis* while diurnal pollinators were seen visiting *S. latifolia*. The latter has been observed in other studies as well (Young 2002). Diurnal pollinators are less effective at transferring pollen between *S. latifolia* than *Hadena* moths (Young 2002, Barthelmess et al. 2006). Based on these observations, it is worth noting that in areas where the two species occur in sympatry a day pollinator is likely to have *S. diclinis* pollen on it, so any visits to an *S. latifolia* are an opportunity to cross-pollinate between the species. In contrast, diurnal pollinators are extremely unlikely to have *S. diclinis* pollen. Furthermore, gene flow within *S. latifolia* populations is attributed to nocturnal pollinators, and diurnal pollinators do not efficiently move pollen between subpopulations (Barthelmess et al. 2006). Thus, while gene flow would be more likely to occur from *S. diclinis* into *S. latifolia,* pollen transfer may be low. Furthermore, in F1 hybrids between *S. latifolia* and *S. diclinis*, there is evidence for significant male inviability and sterility when *S. diclinis* is the paternal parent, suggesting that there may be other isolating barriers at work (Brothers and Delph 2010).

Fitness effects of floral traits

We expected that traits associated with *S. diclinis* would result in higher seed set in plants exposed to diurnal pollinators. In contrast, we predicted that traits associated with *S. latifolia* would have higher seed set in the night treatment. Large flowered plants exposed to pollinators during the day set more seeds than small flowered plants. However, small fruits were also predated less at night ($t = -2.354$, $p = 0.023$; Fig 4b) suggesting that they are advantageous for avoiding predation in *S. diclinis,* which produces far fewer seeds relative to *S. latifolia*. In our experiment we observed that height did not affect seed set. While plants exposed to nocturnal pollinators did not show significant differences in seed set for any of the traits measured, we did find differences in predation rates based on floral traits. Thus, it seems that traits important for pollinator attraction differ from those important for avoiding predation and improving overall plant fitness.

Summary

In F2 hybrids of *S. latifolia* and *S. diclinis* flower height, flower size, and flower color all segregate independently. The height of a plant did not confer higher fitness or influence predation rates, however, flower height significantly improved visitation corresponding to the expected phenotypes for both diurnal and nocturnal pollinators which is similar to results found in other studies (Johnston 1991, Gumbert and Kunze 1999). Flower size was not predictive of visitation by pollinators but increased seed set in plants exposed to diurnal visitors suggesting that large flowers are more attractive to pollinators. However, increased predation on large flowers may result in balancing selection for small flowers in the seed limited *S. diclinis.* In this study, flower color did not appear to influence pollinator visitation, seed set, or predation. While, flower color has been shown to influence pollinators in some systems, other studies have found that color can be influence by pleiotropic effects of other traits important to pollinators (Irwin and Strauss 2003, reviewed in Rausher 2008). Segregation of floral traits revealed several patterns about how pollinators are choosing between plants. These results highlight the complex interactions between plants and their pollinators. Our observation that pure *S.diclinis* are not visited by moths implies that pollinators are limiting gene-flow between *S. diclinis* and *S. latifolia*, however, the cues used by floral visitors to distinguish between the species may include traits not studied here (e.g. scent, ultraviolet color, nectar, etc.) or other ecological factors that influence floral traits.

Selection by pollinators on floral traits may have lead to divergence in some floral traits, however, our results demonstrate that not all traits are important for pollinators, at least at present. The absence of moths visiting *S. diclinis* in our study suggests that there is pre-zygotic floral isolation between these two species, however, it is unlikely that floral isolation is an absolute barrier to gene flow between these species since diurnal pollinators are less discerning. While it appears that pollinators do use flower height and size as cues for visiting, limited conclusions can be drawn about how floral traits affect fitness. Future work should consider additional floral traits that may alter pollinator visitation.

TABLE 1. Principal component loadings.

Flower Size Traits	**PC1**
calyx width	0.779
calyx length	0.842
petal limb length	0.853
cleft length	0.818
Eigenvalue	2.712
% of variance	67.8

Plant HeightTrait	**PC1**
Stem angle	0.872
Plant height	0.872
Eigenvalue	1.52
% of variance	76.1

Color channel	**PC1**
Red reflectance	0.919
Green reflectance	0.894
Blue reflectance	0.906
Eigenvalue	2.645
% of variance	82.2

TABLE 2. Results of a generalized linear mixed model of pollinator visitation. Suites of floral traits contributed to the total number of visits made. Height and shape are principal components that account for multiple correlated traits.

	df	**Coefficient**	**z**	**p**
Intercept	10	-1.76 ± 0.23	-7.76	< 0.001
Height	10	-0.26 ± 0.10	-2.71	0.007
Shape	10	0.20 ± 0.07	3.14	0.002
Number of Flowers	10	0.10 ± 0.02	5.20	< 0.001
Day vs. night	10	0.13 ± 0.22	0.60	0.549
Height* day vs. night	10	0.33 ± 0.12	2.68	0.007
Number of flowers*day vs. night	10	-1.60 ± 0.17	2.54	0.011

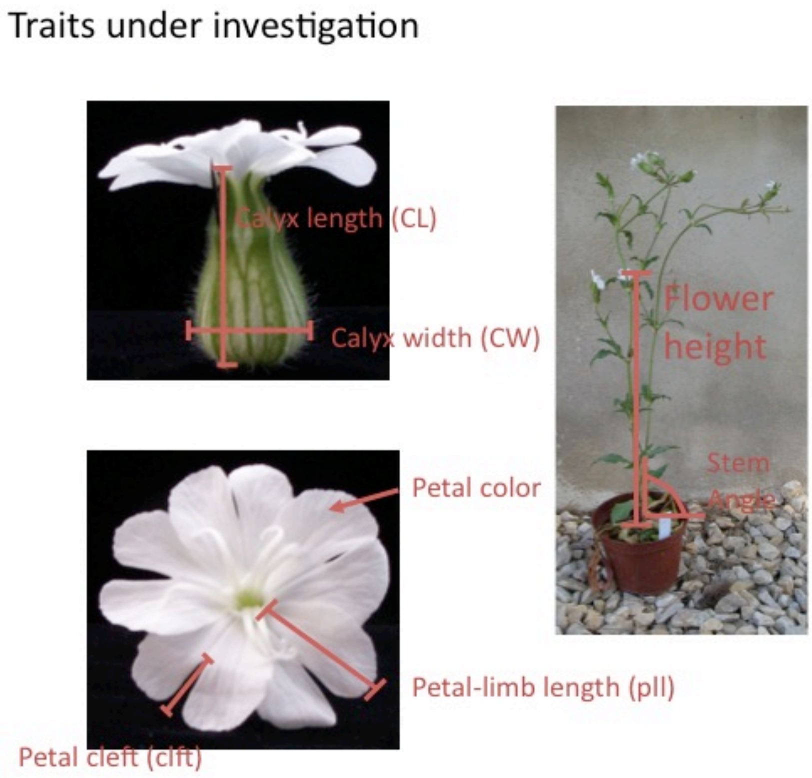
TABLE 3. T-test results comparing plants between the diurnal and nocturnal treatments for total number of flowers, fruits, predated fruits, ovules per fruit, and average seed set.

	df	*Mean ±SE*	*t*	*p*
Average number of flowers per plant	47	15.51±1.18	-0.538	0.593
Average number of fruits set per plant	47	4.66±0.45	-0.348	0.729
Number of predated fruits in treatment group	47	2.75±0.37	-5.424	< 0.001
Average number of ovules per fruit	47	168.0±5.06	-0.252	0.802
Average seed set per plant	47	30.39±3.07	-2.245	0.014

TABLE 4. ANOVA results testing the effect of flower color, height, and size on the average seed set in diurnal and nocturnal arrays. ANOVA results are for Type III sums of squares.

	df	*SS*	*F*	*p*
Day color factor	47	250	1.072	0.306
Day height factor	47	215	0.921	0.342
Day size factor	47	1332	5.702	0.021
Night color factor	47	314	0.211	0.649
Night height factor	47	399	0.268	0.607
Night size factor	47	810	0.543	0.465

a.


Traits under investigation
Calyx length (CL)
Calyx width (CW)
Petal color
Petal-limb length (pll)
Petal cleft (clft)
Flower height
Stem Angle

b.

Figure 1. a). Flower size and flower height measurements are shown. b) Pictured from left to right are a pure *S. latifolia*, two intermediate F2 hybrids, and a pure *S. diclinis*. White flowers have high reflectance values for red, green, and blue. Pink flowers have reduced green and blue values. The principal component factor is also given for each.

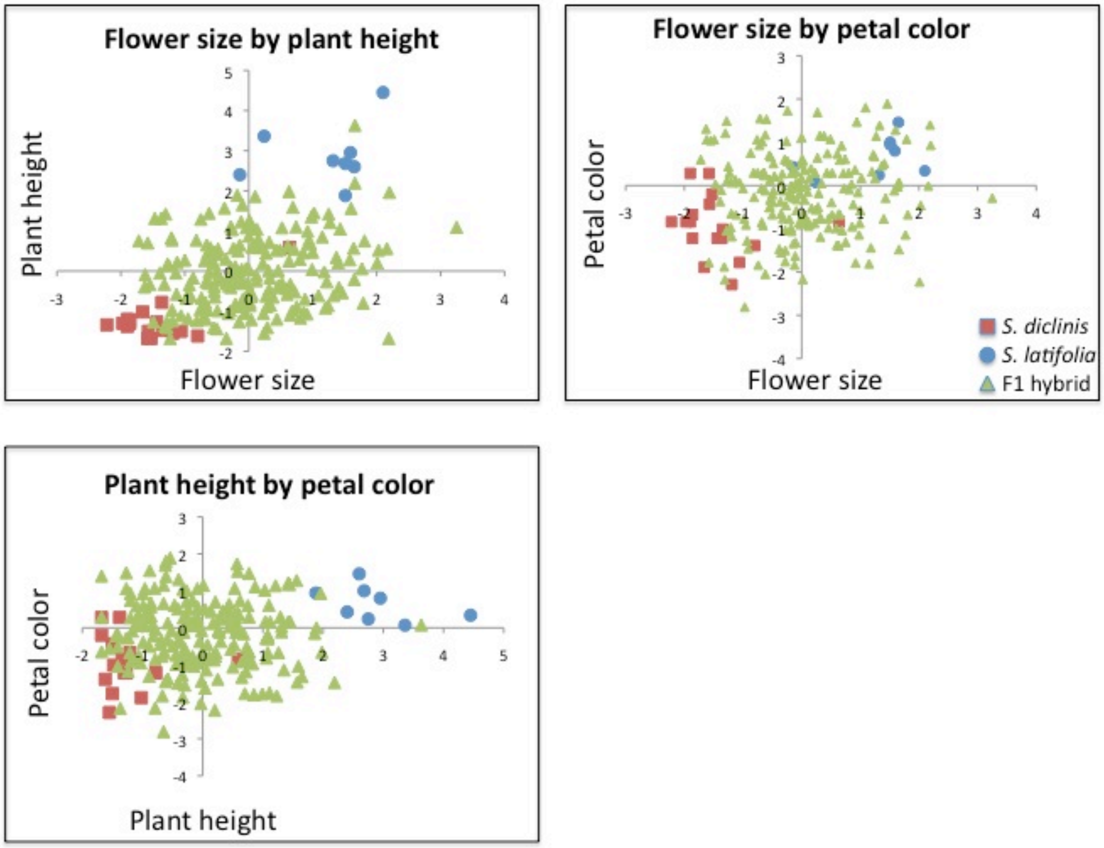


Figure 2. Plant height, petal color, and flower shape/size all segregate independently of each other in F2 hybrids. Hybrids display a wide range of intermediate phenotypes between the two parental species.

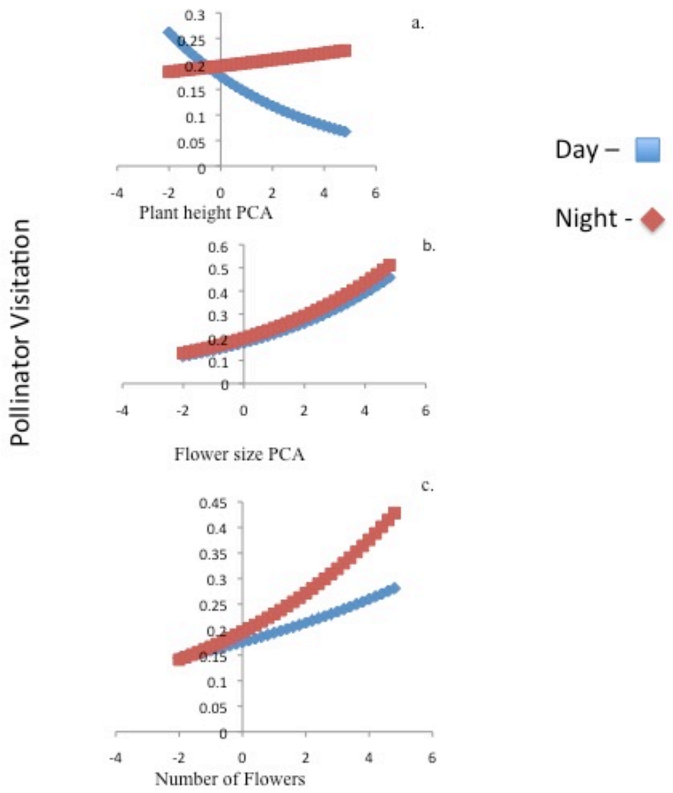


Figure 3. Pollinator visitation based on a) plant height, b) flower size, and c) the number of flowers on a plant.

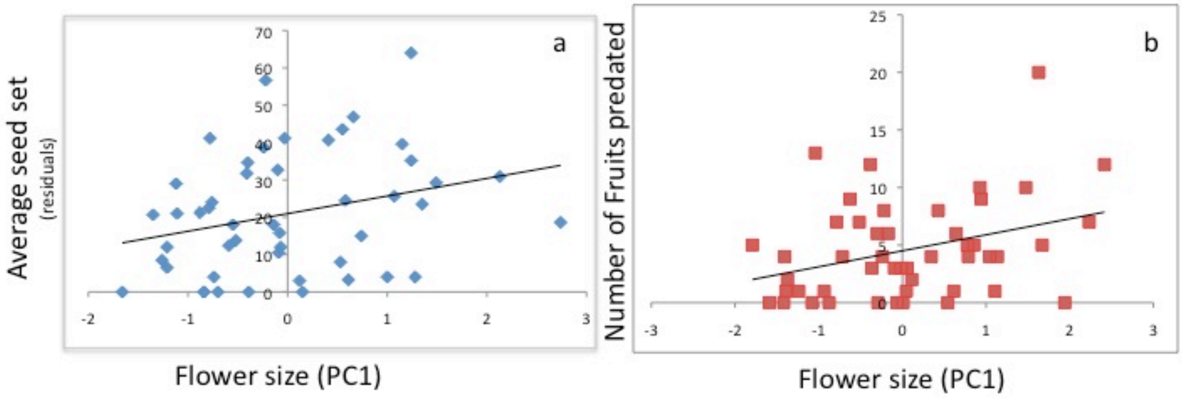


Figure 4. Visitation increased during the day with larger flower sizes as shown by the residuals of average seed set during the day (a). Predation increased at night (b) by flower size PC1.

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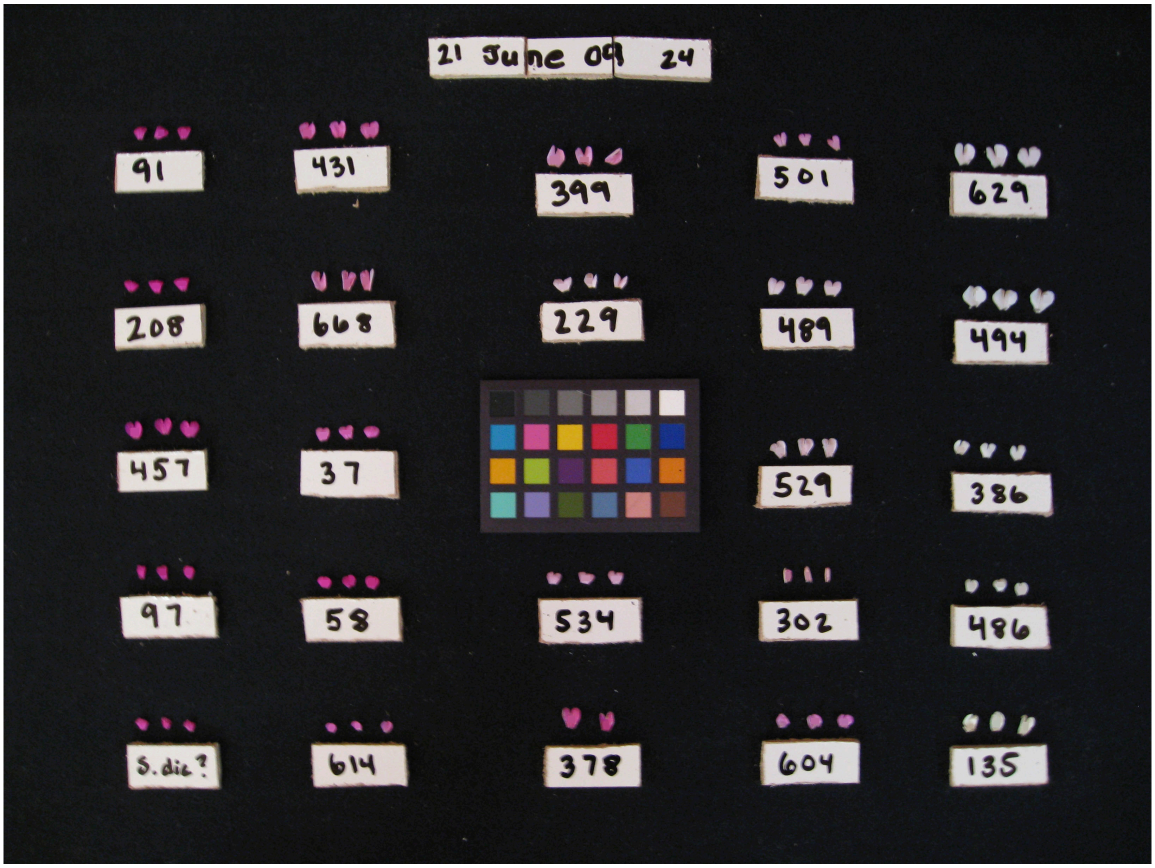

Supplementary Figure 1. Petal color measurements were taken using the GregorMacBeth color checker chart. Petals were arranged (3 per plant) and at least 200 pixels were petal were measured.

CHAPTER 3

HALDANE’S RULE IS EXTENDED TO PLANTS WITH SEX CHROMOSOMES

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ABSTRACT

Haldane’s rule is an empirical phenomenon that has been observed in animals with sex chromosomes. The rule states that the heterogametic sex (XY or ZW) will be "absent, rare, or sterile" following hybridization between two species. Despite the near ubiquity of Haldane’s rule in animal hybridizations, it has not been documented in organisms other than animals. Here we show evidence for both rarity and sterility in hybrid male but not female offspring in crosses between three dioecious plant species from the genus *Silene* with heteromorphic (XY) sex chromosomes. Our results are consistent with Haldane’s rule, extending its applicability to plants with sex chromosomes.

INTRODUCTION

J.B.S. Haldane was the first to report that when animals are hybridized, the heterogametic sex (i.e., XY or ZW) is more likely to be absent, rare, or sterile relative to the homogametic sex (Haldane 1922). Since then, this phenomenon has been seen in numerous animal taxa, including insects, mammals, birds, and reptiles (Laurie 1997; Coyne and Orr 2004). Interestingly, Haldane's rule is observed regardless of which sex is heterogametic, implying that incompatibilities involving the sex chromosomes contribute disproportionately to post-zygotic reproductive isolation (Laurie 1997; Coyne and Orr 2004). Even animals with non-heteromorphic X and Y chromosomes commonly exhibit Haldane's rule for sterility, although not for absence or rareness (commonly termed inviability) (Presgraves and Orr 1998). Results such as these suggest that X chromosome hemizygosity is an important underlying mechanism for inviability in the heterogametic sex, and moreover, that more than one mechanism is likely to underlie the phenomenon.

The presence of sex chromosomes in some dioecious plant taxa allows for an independent test of Haldane's rule. However, plants differ from animals in ways that make it less likely for Haldane's rule to be observed. For example, the Y chromosomes of plants can be relatively young (e.g., 5-10 my old in *S. latifolia* (Nicolas et al. 2005)), express their genes prior to fertilization (Haldane 1932; Erickson 1990), and thus are unlikely to have degenerated to the extent of Y's in many animals (240-320 my old), such as some *Drosophila* and mammals (Lahn and Page 1999; Nicolas et al. 2005). Plant Y chromosomes are often not diminished in size relative to the X and in most cases are the largest of the chromosomes (Ainsworth 2000). As a consequence of these attributes, the sex chromosomes of plants are likely to be more similar in gene content than those of most animals Haldane's rule has been observed in. Hybrids are therefore less likely to exhibit the hemizygosity required for the most widely accepted genetic model for inviability (Read and Nee 1991; Presgraves and Orr 1998; Coyne and Orr 2004). Thus, the genetic and evolutionary dynamics governing inter-specific incompatibilities may differ between plants and animals with separate sexes. Despite these differences, evidence of Haldane's rule in dioecious plants would suggest a fundamental role for sex chromosomes as the cause of inviability and sterility in heterogametic hybrids (Coyne and Orr 1998, Noor and Feder 2006).

Dioecious plants from the genus *Silene* are ideally suited for testing Haldane's rule in plants. *Silene latifolia*, *Silene dioica*, and *Silene diclinis* are three closely related species with sex chromosomes, in which males are the heterogametic sex. These three species are thought to have shared a common ancestor with sex chromosomes (Desfeux et al. 1996), with *S. latifolia* and *S. dioica* being more closely related than either is to *S. diclinis* (B. Oxelman, personal communication). Furthermore, the sex chromosomes of *Silene* have been well studied (especially those of *S. latifolia*; e.g., Lengerova et al. 2003). In addition to being relatively young, all Y-linked copies of genes of *S. latifolia* discovered so far are functional, with one exception (Nicolas et al. 2005, Bergero et al. 2007). Hence, homology between the X and Y may be relatively high compared to those of many animals. Additionally, despite the Y chromosome of *S. diclinis* having been split in two by reciprocal translocation with an autosome (Howell et al. 2009), viable hybrid seeds can be produced from crosses between all three species.

Experimental demonstration of Haldane's rule in animals is thought to have spawned a renaissance in speciation genetics (Laurie 1997; Presgraves 2002; Coyne and Orr 2004). However, conformity with Haldane's rule has never been tested in any organism other than animals; its significance and generality depends on documentation in other major taxonomic groups (Read and Nee 1991; Laurie 1997; Presgraves 2002; Coyne and Orr 2004). Determining whether Haldane's rule extends beyond animals to plants with heteromorphic sex chromosomes may help to elucidate whether Haldane's rule is a general phenomenon across taxa or a pattern of speciation that only applies to animals.

MATERIALS AND METHODS

Crossing Design

Silene latifolia and *S. dioica* are widespread throughout Europe and hybrids between them have been found in some areas of sympatry (Baker 1948; Goulson and Jerrim 1997). In contrast, *S. diclinis* is an endangered, narrow endemic, occurring only in the Valencia region of southeast Spain (Prentice and Andersson 1997; Montesinos et al. 2006). It occurs in sympatry with *S. latifolia*. The two species differ markedly for many morphological traits (*S. latifolia* is upright, relatively tall and produces flowers that open at night and *S. diclinis* is prostrate and produces bright pink flowers that open during the day) and phenotypically detectable hybrids (which are intermediate for the above mentioned traits) have not been observed in the field (Prentice 1976, A. Brothers, personal observation). All of the populations used in this study are allopatric.

Seeds from several plants per species (from a population in France for *S. latifolia*, France for *S. dioica*, and Spain for *S. diclinis*) were grown to flowering in a greenhouse at Indiana University. Five males and five females from each species were selected as parents and used to perform crosses. Nine types of crosses were produced by hand pollinating flowers: three intra-specific crosses and six hybrid crosses, in which each species was crossed to the other two species as both the mother and the father. Seeds were collected from the resulting fruits and planted, for a total of 6750 seeds (9 types of cross x 5 families/cross x 150 seeds/family). The sex of each plant was determined upon flowering.

Fertility Measures

To estimate ovule viability, we cut open fresh flowers from both pure species and hybrid females, removed all the ovules we considered fully developed and viable, and then stained them with a tetrazolium salt (methyl-thiazolyl blue, MTT). The area of the ovule containing the egg cell stains a light purple if viable. This step confirmed our ability to count ovules as viable vs. non-viable. Viable ovule number per flower was chosen as the measure of fecundity for females, in order to provide the most quantitative measure possible. We chose this measure based on the following: 1) F_1 females readily set seed when crossed (and seed production by these females was intermediate in value between the two pure species), 2) this measure removes any later-generation incompatibilities that may arise when hybrids are crossed to each other or back to the pure species, and 3) ovule number and seed production are highly correlated. In order to show the latter correlation we crossed a total of 60 pure-species and F_1 females (from *S. latifolia* x *S. diclinis* crosses and *S. diclinis* x *S. latifolia* crosses) to F_1 males and pure-species males. We allowed the fruit to fully mature, and counted the number of fully developed seeds within the fruit (from 1 to 5 fruit per female; $N = 178$ fruits). Ovule number was highly correlated with the number of viable seeds that a flower produces (Pearson's correlation = 0.91, $P = 0.043$). In order to obtain data on viable ovule number, the third flower to open on each female was collected, preserved in 70% ethanol, later dissected, and the number of fully developed ovules was counted under a dissecting microscope.

To quantify male fertility, we counted pollen grains per flower and measured the proportion of grains that were viable. Two anthers were harvested from the third flower to open on each male. One was used to count pollen grains using an ELZONE II particle analyzer (Micromeritics, Norcross, GA). The other was preserved in lactic-acid glycerol for estimating pollen viability under a compound microscope. Staining methods proved to be inconclusive. Hence, pollen viability was based on size. Pollen grains of various sizes were germinated on agar media from both pure species and F_1 males and the threshold size for germination was determined. Pollen grains below this threshold size (< 0.03 mm) were considered inviable. Moreover, grains below this size tended to be shriveled when viewed under the microscope. Fertility measures were taken from a random subset of approximately 50 individuals from each sex for each cross (~10 individuals/sex per family).

Analyses

To determine whether male progeny from hybrid crosses between *S. latifolia*, *S. diclinis*, and *S. dioica* were less abundant than expected, we determined whether the sex ratio from hybrid crosses was significantly female biased relative to the mid-parent value of the two parental species. We took this approach rather than using 50:50 as our expected value, because *S. latifolia* has been shown to have female-biased primary sex ratios (Taylor 1994a). Haldane's rule for sterility predicts that if hybrid females will not show a reduction in their fertility relative to pure species females, whereas hybrid males will. Genes of any one of the species controlling ovule number or pollen production/viability might be dominant in a hybrid cross, thereby pulling the mean closer to the mean of that species (i.e., less than complete additivity). Hence, our criterion for inferring Haldane's rule for sterility was that the fertility of female hybrids be above the lower of their parental means, whereas the fertility of male hybrids be significantly below the lower of their parental means.

Chi-Squared tests were used to determine whether the expected number of males differed significantly from the observed number of males (df = 1). Intra-specific crosses were tested against the expectation of a 50:50 sex ratio, whereas the observed sex ratios of intra-specific crosses were used as the expected values for hybrid crosses. One-way ANOVAs followed by linear contrasts were used to compare the number of ovules per flower, the number of viable pollen grains produced per flower, and the proportion of those pollen grains that were viable.

RESULTS

Our results are consistent with Haldane's rule for either rarity and/or sterility for five of the six hybrid crosses. In hybrid crosses using *S. dioica* as the mother, we found a significant reduction in the number of F_1 males relative to intra-specific crosses. This reduction occurred regardless of which of the other two species was the father. Similarly, hybrid crosses using *S. diclinis* as the father also resulted in significantly fewer males than expected in the F_1 generation, regardless of which of the other two species was the mother. In total, three of the six hybrid crosses resulted in a significant reduction in the proportion of male progeny produced (Fig. 1, Table 1).

Haldane's rule was most pronounced for hybrid sterility. Ovule counts per flower for all six hybrid crosses were above the lower of the parental means (Fig. 1). In contrast, hybrid males often showed a reduction in pollen fertility relative to males from intra-specific crosses (Fig. 1, Table 1). The total number of viable pollen grains produced per flower was significantly lower than that of the lower parental mean in four out of six hybrid crosses. This reduction in male fertility was largely caused by the proportion of pollen grains that were viable being significantly lower than the lower parental mean in five out of six hybrid crosses.

DISCUSSION

We found evidence for Haldane's rule in crosses between three species of dioecious *Silene* with heteromorphic sex chromosomes. For each species-pair cross, fewer male progeny than expected were seen for one direction of the cross, but not the other. Results were more pronounced for sterility, as five of six hybrid crosses showed evidence of male sterility. Only the *S. latifolia* x *S. dioica* cross failed to conform, although even in this case one measure of male sterility was in the expected direction (Fig. 1). Previously, crosses between *S. latifolia* and *S. dioica* were performed in a study investigating Y linkage of sex-ratio distorters (Taylor 1994b). Analyses of hybrid F_1 sex ratios were not reported in that study, as the emphasis was on comparing the F_2 generation with the grandparental generation. However, analyses were performed on hybrid F_1 sterility. While they reported no reduction in pollen production for their hybrid males (whereas we did for the cross in one direction, but not the other), their *S. dioica* x *S. latifolia* males showed a reduction in pollen viability similar to what we found. Together, these findings concur with studies of hybrid zones in nature between *S. latifolia* and *S. dioica*, in which introgression is more common when *S. dioica* is the father and *S. latifolia* is the mother, although there is no evidence of genome-wide admixture (Minder 2007, Karrenberg and Favre 2008).

Our results mirror those of many previous studies showing Haldane's rule in animals. For example, asymmetry in inviability, in which male frequency is significantly reduced in one cross but not the reciprocal cross, is sufficiently common among crosses between animal species to have been coined "Darwin's corollary" (Turrelli and Moyle 2007). In addition, previous studies of animals found that male sterility evolves faster than inviability (Tao and Hartl 2003; Wu et al. 1996), and our results support this premise.

Several theories regarding mechanism have been put forward to explain Haldane's rule (reviewed in Coyne and Orr 2004; Presgraves 2008). The most widely accepted theory is the dominance theory, which applies to both inviability and sterility, and involves incompatibilities caused by the relative hemizygosity of loci on the sex chromosomes combined with recessivity or partial recessivity of these loci. For example, *Drosophila* species with highly degenerate Y chromosomes, often show hybrid male inviability (Coyne and Orr 2004; Laurie 1997). In contrast, mosquitoes in the genus *Aedes*, which have Y chromosomes that contain only a small region that differs from the X, often display hybrid male sterility but not inviability, leading to the conclusion that there is insufficient hemizygosity in the heterogametic sex to cause inviability (Presgraves and Orr 1998). Dioecious *Silene* Y chromosomes are likely intermediate between the non-degenerate state of *Aedes* Y's and the highly degenerate Y's of *Anopheles*, *Drosophila*, and mammals (Presgraves and Orr 1998; Coyne and Orr 2004; Nicolas et al. 2005; Bergero et al. 2007). This premise is based not only on the relatively large size of the *S. latifolia* Y chromosome and its relatively young age, but also on the fact that most X-linked genes discovered so far are functional on the Y (Nicolas et al. 2005, Bergero et al. 2007) and QTL for several important fitness traits are located in the recombining portion of its Y (Scotti and Delph 2006). The highly female-biased sex ratios demonstrated here for hybrid *Silene* suggest that sufficient hemizygosity occurs well before full degeneration of the Y chromosome and/or other mechanisms are operating to skew sex ratio.

An alternative mechanism for skewed sex ratios is the meiotic-drive theory, which involves a mismatch between meiotic drivers and their suppressors (Frank 1991; Hurst and Pomiankowski 1991). Results consistent with the presence of drivers of sex ratio and their suppressors have been observed for *S. latifolia* and *S. dioica* (Taylor 1994a,b), and it is notable that even our intra-specific crosses with *S. latifolia* and *S. diclinis* were significantly female biased (Table 1). It thus seems plausible that incompatibilities between meiotic drivers and suppressors of the different species could be involved in the observed asymmetry of sex ratio in hybrids.

A third theory for Haldane's rule applies solely to hybrid male sterility. The faster-male theory is based on the premise that genes affecting male fertility will evolve more quickly and/or be more susceptible to disruption than genes affecting female fertility (Wu and Davis 1993). Faster-male evolution could occur in plants, especially given that the expression of genes in pollen provides for selection on male-fertility genes (Delph et al. 1998). Hence, the male sterility found in our hybrids may be the result of pollen being inherently more sensitive developmentally than ovules and/or the genes controlling aspects of pollen having evolved more quickly (Wu and Davis 1993), rather than heterogamety *per se*. A similar conclusion was reached in a study of *Aedes* mosquitoes, which exhibited hybrid male sterility in spite of very limited differences between the X and Y chromosomes (Presgraves and Orr 1998). Nevertheless, while skewed sex ratios were not seen in the *Aedes* study, we observed them here and faster-male evolution cannot account for inviability, only sterility. While limited conclusions can be drawn about the causes of Haldane’s rule from our study, the ease of crossing between generations and species suggest that future plant studies will uncover the specific genetic mechanisms involved in the observed inter-specific incompatibilities.

In conclusion, our results demonstrate that post-zygotic barriers contribute to reproductive isolation between closely related plant species with heteromorphic sex chromosomes. Moreover, the relatively young age of the sex chromosomes in these plants demonstrates that both hybrid inviability and sterility can occur early in the differentiation of sex chromosomes and that Haldane’s rule is likely an early step in the speciation process. Lastly, our findings enhance the hypothesis that Haldane's rule really is a rule; that is, that it represents a pattern of broad generality.

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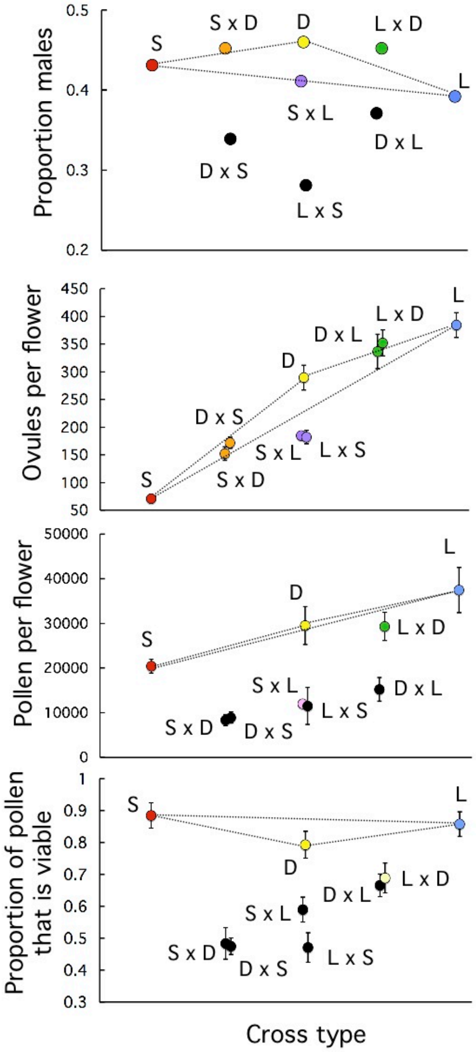
Table 1. Results of analysis on sex ratio (χ^2) and male fertility measures (linear contrasts).

Sex Ratio	χ^2	P
Hybrid crosses		
L x D**	1.50	0.222
L x S	14.44	<0.001
D x L	4.14	0.042
D x S	7.43	0.006
S x L	0.001	0.971
S x D	0.09	0.763
Intra-specific crosses		
L x L	23.95	<0.001
D x D	2.93	0.087
S x S	12.60	<0.001

# of pollen grains/flower	$F_{1, 36}$	P
S x D vs. S	7.77	0.008
D x S vs. S	7.10	0.011
S x L vs. S	3.81	0.059
L x S vs. S	4.26	0.046
D x L vs. D	10.91	0.002
L x D vs. D	<0.01	0.962

Proportion of pollen grains that are viable	$F_{1, 36}$	P
S x D vs. D	31.11	<0.001
D x S vs. D	33.04	<0.001
S x L vs. L	23.40	<0.001
L x S vs. L	48.95	<0.001
D x L vs. D	5.24	0.028
L x D vs. D	3.51	0.069

Figure 1. Haldane's rule for inviability and sterility in hybrid males, as illustrated with sex ratios and fertility measures from the nine types of crosses. Circles filled with primary colors are used to represent the means obtained from intra-specific crosses: S = *S. diclinis* (red), D = *S. dioica* (yellow), L = *S. latifolia* (blue). Colors representing hybrid crosses (mother x father) are based on the mixture of the two primary colors of the parent species involved in the cross (red x yellow = orange, red x blue = purple, yellow x blue = green). Black circles represent means that are significantly lower than the lowest parental mean, and indicate conformance with Haldane's rule. The pink and pale yellow circles (see the lower two panels) represent means that are only marginally significantly lower than the lowest parental mean (Table 1). (a) Hybrid inviability depicted as the proportion of males relative to females. (b) Female fertility - the number of ovules produced per flower for females (mean ± 1SE). (c) Male fertility - the total number of pollen grains produced per flower (mean ± 1SE), calculated as the number of pollen grains produced multiplied by the proportion that were viable. (d) The proportion of pollen grains per flower that are viable (mean ± 1SE).


Proportion males
0.5
0.4
0.3
0.2
S
S x D
D
L x D
L
S x L
D x L
D x S
L x S
Ovules per flower
450
400
350
300
250
200
150
100
50
L
L x D
D x L
D
D x S
S x L
L x S
S
S x D
Pollen per flower
50000
40000
30000
20000
10000
0
L
D
S
L x D
S x L
D x L
L x S
S x D
D x S
Proportion of pollen that is viable
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
S
L
D
L x D
D x L
S x L
S x D
D x S
L x S
Cross type

CHAPTER 4

THE FATE OF NEO-SEX CHROMOSOMES IN HYBRIDS OF DIOECIOUS SILENE WITH HETEROMORPHIC SEX CHROMOSOMES

Amanda N. Brothers, Victoria N. Jideonwo, and Lynda F. Delph

ABSTRACT

Within the genus *Silene*, section Elisanthe, there are three species with heteromorphic sex chromosomes. Previously, the chromosomes of all three species were thought to be similar, however, it was recently reported that *S. diclinis* has a set of neo-sex chromosomes as the result of a translocation between the Y chromosome and an autosome. Furthermore, previous work in this system suggested that crosses between *S. diclinis* and *S. latifolia* did not produce viable hybrids (Prentice 1976). We have successfully produced viable hybrids in these two species. Here we use solid-staining techniques, to show that hybrids between *S. latifolia* and *S. diclinis* (with *S. diclinis* as the paternal parent), produce karyotypes similar to those seen in *S. diclinis,* demonstrating that neo-sex chromosomes can be inherited across species. We also explore methods for how fluorescence *in Situ* hybridization (FISH) can be applied to studies in dioecious *Silene* with sex chromosomes. While neo-sex chromosomes may contribute to reproductive isolation between these two species, it is unlikely that they drove speciation.

INTRODUCTION

The role of newly formed sex chromosomes in speciation events between closely related species has only recently been addressed. For example, in stickleback fish it was found that a relatively young neo-sex chromosome contributed to speciation because genes found on the new sex chromosome were important for courtship display, which altered mate choice (Kitano et al. 2009). Furthermore, it was hypothesized that selection for linkage between male-beneficial traits and the sex-determining region promoted the fixation of the neo-sex chromosome arrangement (Kitano et al. 2009). In a recent study by Howell et al. (2009), it was reported that the dioecious plant, *Silene diclinis*, contains a pair of neo-sex chromosomes and that the neo-sex chromosomes might be an important reproductive isolating barrier between *S. diclinis* and the closely related species, *S. latifolia*. Although, in previous studies of crosses between *S. latifolia* and *S. diclinis* viable hybrids were rare (Prentice 1976), more recent work has shown that these two species readily hybridize in greenhouse conditions with hand pollinations (Brothers and Delph 2010). Here, we examine the karyotypes of *S. latifolia*, *S. diclinis*, and F1 hybrids between the two species to determine whether neo-sex chromosomes can be inherited across species and consider their importance in reproductive isolation between these two species.

Proper chromosome segregation during meiosis is important for the correct distribution of genetic material in the majority of plants and animals. Alterations in chromosome structure caused by duplications or translocations are often lethal or can affect fertility. However, reciprocal translocations between chromosomes can result in stably inherited quadrivalents or circular formations when synapsis or side-by-side pairing of homologous chromosomes occurs. The most well known example of ring-structures are found in *Oenothera* where several chromosomes, and sometimes all of the chromosomes, form a single ring (Cleland 1967). Stable sex-linked chains are found in animals including platypus and a variety of invertebrates (Gruetzner et al. 2004, Gruetzner et al 2006). In organisms with sex chromosomes, reciprocal translocations between the sex chromosomes and autosomes can result in decreased recombination and the formation of neo-sex chromosomes.

Because the majority of plant species are hermaphroditic, only a small fraction (~6%) of plant species have evolved sex chromosomes (Ming and Moore 2007). However, within the genus *Silene* section Elisanthe, three species are dioecious with sex determined by heteromorphic sex chromosomes. Female plants are homogametic (XX) and males are heterogametic (XY). The sex chromosomes of *Silene* are relatively young (approximately 5-10 my old) relative to those found in mammals (240-320 my old) (Nicolas et al 2005). The sex chromosomes of *Silene latifolia* have been relatively well studied compared to other plant species and provide an ideal system in which to test questions regarding plant sex-chromosome evolution (Bernasconi et al. 2009). *Silene latifolia* is one of the few plant species with cytologically distinguishable sex chromosomes (Armstrong and Filatov 2008). The Y chromosome found in males is the largest of the chromosomes and accounts for approximately 9% of the total genome and is slightly larger than the X chromosome, which is second in size (Reviewed in Vyskot and Hobza 2004). While the Y chromosome of *S. latifolia* has been extensively studied (Armstrong and Filatov 2008), the Y chromosomes of the closely related dioecious species *S. dioica* and *S. diclinis* have received less attention. However, recently it was reported that *S. diclinis* has a stable sex-linked chain quadrivalent structure composed of the X chromosome, the Y chromosome, and a set of neo-sex chromosomes (Fig. 1) (Howell et al. 2009). This is thought to have occurred as the result of a reciprocal translocation between an autosome and the Y-chromosome resulting in a neo-Y or Y2 chromosome (Howell et al. 2009). Consequently, a previously normal autosome now co-segregates with the X chromosome in F1 females and is referred to as the neo-X. The X chromosome, Y chromosome, neo-X, and Y2 chromosome form a stably inherited quadrivalent during cell division with the two homologous chromosomes pairing side by side (Fig 1). This means the Y2 chromosome is always inherited with the Y chromosome. In *S. diclinis*, the Y chromosome, the X chromosome, and the Y2 chromosome are all similar in size and larger than the autosomes (Howell et al. 2009). Previous work with *S. diclinis* had shown that the X and Y chromosome were similar in size although a third large chromosome was not observed (Van Nigtevecht and Prentice 1985). Howell et al. (2009) found that the neo-sex chromosomes of *S. diclinis* were found throughout its range, but neo-sex chromosomes have not been found in other closely related species of *Silene* suggesting that the reciprocal translocation occurred within the last 1-2 my (Howell et al. 2009).

Silene diclinis is an endangered, endemic species found only in the Valencia region of Spain. The entire range of *S. diclinis* is found within an 18 x 9 kilometer area and there are few populations remaining. *Silene latifolia* occurs throughout Europe and occurs sympatrically with *S. diclinis*. Crosses between the two species have been successful in greenhouse studies, however males suffer both higher inviability and higher sterility (i.e., Haldane's rule) in the F1 hybrid generation (Brothers and Delph 2010). Phenotypically detectable hybrids between the two species have not been observed in the wild (Brothers, pers. obs.). While the sex chromosomes of *S. latifolia* have been well characterized, work in *S. diclinis* is less extensive. However, some of the molecular-genetic methods developed in *S.*

latifolia can be applied to *S. diclinis* (Howell et al. 2009). This system provides an opportunity to examine the fate of neo-sex chromosomes when crossed with a closely related species without neo-sex chromosomes.

Although it has been suggested that the absence of hybrids between *S. diclinis* and *S. latifolia* in the field are the result of chromosomal incompatibilities between the two species, this theory does not explain the ease with which hybrids can be generated in the greenhouse. This study outlines the initial procedures for mapping the sex chromosomes and neo-sex chromosomes in hybrids between *S. latifolia* and *S. diclinis*. We provide detailed methods for how chromosome squashes and FISH techniques can be applied to *Silene* and suggest how future studies should proceed. We also draw conclusions about the segregation of neo-sex chromosomes based on solid-staining techniques.

METHODS

Plant preparation

Pure species *S. latifolia*, *S. diclinis*, and F1 hybrids were raised under standard greenhouse conditions at Indiana University. When plants reached maturity and sex could be determined, plants were transferred to larger pots and placed in a mixture of loose soil, vermiculite, and Metro Mix™. After one week, fine root hairs were harvested (1-2 cm), washed in dH20, and placed in 0.65ml eppendorf tubes with holes in the top (~6 root tips/tube) and misted with water. The tubes containing the roots were dropped into a gas chamber (Murr Instrument Shop, Columbia Missouri) and treated with nitrous oxide (160 PSI) to stall cells in the metaphase stage. After at least 2 hours, roots were removed from the nitrous-oxide treatment, and washed with glacial acetic acid (90%) for ten minutes. The acetic acid was removed and chilled 70% ethanol was added to each tube. This step was often repeated, especially when the roots were soiled. Treated root tips were then placed in clean 0.65 ml eppendorf tubes containing 70% ethanol and stored at 4° for up to several months. Roots were removed from cold storage for karyotyping as needed.

Root tips were selected by viewing under a dissecting scope (Zeiss). Appropriate roots exhibit bright white, dense tips. Root tips were washed with 1X citric buffer, trimmed to 1mm and placed into an enzyme solution of pectolyase and cellulase and digested in a 37° water bath with digest times ranging from 10-30 minutes. Immediately upon removal from the water bath, the roots were placed in ice. Using a metal probe, roots were smashed until the enzyme solution appeared cloudy. The tubes were then rinsed two times with ethanol. Between each rinse, the tubes were centrifuged to allow the pellet to adhere to the wall of the tube. After the final washing, tubes were rolled and excess ethanol was poured off and the tubes were allowed to dry. The remaining pellet was then suspended in 10 ul of a 90% glacial acetic acid and methanol solution. Two slides were made from each tube by dropping 5ul of the glacial acetic acid-methanol solution onto clean slides and drying for several hours in a humidity chamber (box lined with damp paper towels and covered by damp paper towels). We observed the initial karyotypes by examining slides at 20X using light microscopy. Alternatively, coverslips can be mounted in DAPI with vectashield (Vector) if only karyotypes are to be observed without hybridization. To observe the genic probes we continued with the fluorescent in situ hybridization (FISH) procedures as described below.

FISH probes preparation

Two genic probes were used, SlY1 and SlY4. We selected regions from the sequenced genes of SlY1 and SlY4 from GenBank and made primers that were within the proper range of 200-500 base pairs for using a digoxigenin labeling probe (Table 1). DNA was extracted from male *S. latifolia* plants and amplified using PCR. The PCR parameters were as follows: SlY1 – 94°C for 2:00, 29 × [94°C for 0:30, 56°C for 0:45, 72°C for 2:00], 72°C for 5:00 (Moore et al 2003); SlY4 – 95°C for 3:00, 3 × [95°C for 0:30, 65°C for 0:30, 72°C for 2:00], 3 × [95°C for 0:30, 62°C for 0:30, 72°C for 2:00], 27 × [95°C for 0:30, 59°C for 0:30, 72°C for 2:00], 72°C for 4:00 (Atanassov et al. 2001). The SlY1 probe labels the Y chromosome and the X chromosome and the SlY4 probe labels the neo-Y chromosome. The probes were created by nick translation (Roche). To label the PCR product with digoxigenin-11-dUTP (Roche), water was added to1 μg of the PCR product for a total volume of 16μl. Then 4 μl of Nick translation mix (Roche) was added to the PCR product and the water (total volume of 20 μl). The mixture was incubated at 15°C for 2 hours and then the reaction was stopped by the addition 1 μl of 0.5M EDTA (pH 8.0) and heated to 65° for 10 minutes. The probe was then stored at -20°C and used as needed.

Slide Hybridization

Slides with good chromosome squashes were selected for hybridization tests. First, slides were incubated for 30 minutes on a 60°C heating block. They were then treated with 60 μl RNase A and incubated for 1 hour at 37°C in a box lined with tissue moistened with 2X SSC. The slides were dehydrated in a series of alcohol washes (70%, 90%, 100%) for two minutes each. Hybridization buffer was prepared and 3 μl of the probe nick translation mix was added to 17 μl of hybridization buffer for each slide. The probe nick translation mix was applied directly to the slides and then the slides were heated to 80°C for four minutes and then placed in a moist chamber at 37°C for at least 15 hours. Following the incubation period, the slides were treated with blocking buffer (5% non-fat dry milk in 4X SSC) followed by a thirty-minute incubation at 37°C. The slides were washed with TBS-Tx ten times and incubated for an additional 30 minutes with the anti-digoxigenin-rhodamine (suspended in Abdil), which is used to detect the probe. Following the incubation period, the slides were washed again with TBS-Tx ten times and then mounted with the counterstain DAPI in vectashied (Vector). The slides were then viewed under a Nikon E800 fluorescent microscope using the Metamorph imaging program.

RESULTS

Previously it was shown that *S. diclinis* males have three sex chromosomes and a neo-X chromosome (Howell et al. 2009). The Y chromosome, the X chromosome, and the Y2 chromosome were all found to be of similar size, while the neo-X chromosome was found to be small and indistinguishable from the autosomes based on size, and is not thought to be functionally different from other autosomes (Howell et al. 2009). Here, we found that three large chromosomes were present in F1 hybrids of *S. latifolia* and *S. diclinis*, demonstrating that the Y2 chromosome is present in F1 hybrid males.

Solid-staining techniques revealed that F1 hybrids between *S. latifolia* and *S. diclinis* had 2n=24 chromosomes as expected. In F1 hybrids, there appeared to be three large chromosomes per karyotype, similar to those seen in pure *S. diclinis* spreads. The Y chromosome, the X chromosome, and the neo-Y chromosome (Y2) (Fig. 2) have all been reported to be approximately the same size in *S. diclinis* (Howell et al. 2009) and are most likely the three large chromosomes observed in our spreads. While genic probes would be more conclusive, the probes used in this study resulted in non-specific labeling (Fig. 2). Consequently, we were not able to definitively identify the sex chromosomes or the neo-sex chromosomes with FISH techniques. However, solid staining-techniques suggest that chromosomal segregation in hybrids is comparable to that observed in pure *S. diclinis* individuals.

DISCUSSION

Chromosomal rearrangements are known to be important for sex-chromosome evolution. For example, inversions on the human Y chromosome are thought to have suppressed recombination, enabling differentiation between the X and Y chromosomes (Lahn and Page 1999). Similarly, within *S. latifolia*, two large inversions on the Y chromosome have reduced recombination and likely are responsible for the increased number of repetitive sequences on the Y chromosome (Hobza et al. 2007). In *S. diclinis*, the reciprocal translocation between the Y chromosome and an autosome has resulted in a pair of neo-sex chromosomes (Fig. 1) (Howell et al. 2009). Chromosomal rearrangements in *S. diclinis* have been reported as a potential cause of speciation between *S. latifolia* and *S. diclinis* (Howell et al. 2009). This study suggests that the Y2 chromosome is inherited in males in the F1 generation in the same way it is in pure species *S. diclinis* and does not act as a complete reproductive isolating barrier. These findings imply that neo-sex chromosomes can be inherited across species. Furthermore, hybrids between *S. latifolia* and *S. diclinis* are capable of producing viable gametes and offspring (Brothers and Delph 2010), despite the neo-sex chromosomes found in *S. diclinis*, demonstrating that complete genetic isolation has not yet occurred. Whether the Y2 chromosome contains sex-determining genes is not yet clear. Consequently, it is possible that the advent of the neo-sex chromosomes has reduced recombination between Y2 and the neo-X in the new arrangement (Howell et al. 2009). It is also possible that the neo-sex chromosome arrangement confers fitness advantages within *S. diclinis* that are associated with particular genes being linked that are not beneficial in *S.*

latifolia. For example, in threespine sticklebacks, it was found that selection favors a chromosomal fusion between the sex-determining region and traits important for courtship display leading to the formation of neo-sex chromosomes (Kitano et al. 2009). Sex-chromosome divergence between closely related species warrants further investigation. It is surprising that hybrids are viable; however, determining the importance of chromosomal rearrangements in the context of speciation will require documentation of the fitness consequences of neo-sex chromosomes, as well as a better understanding of other isolating barriers at work in this system.

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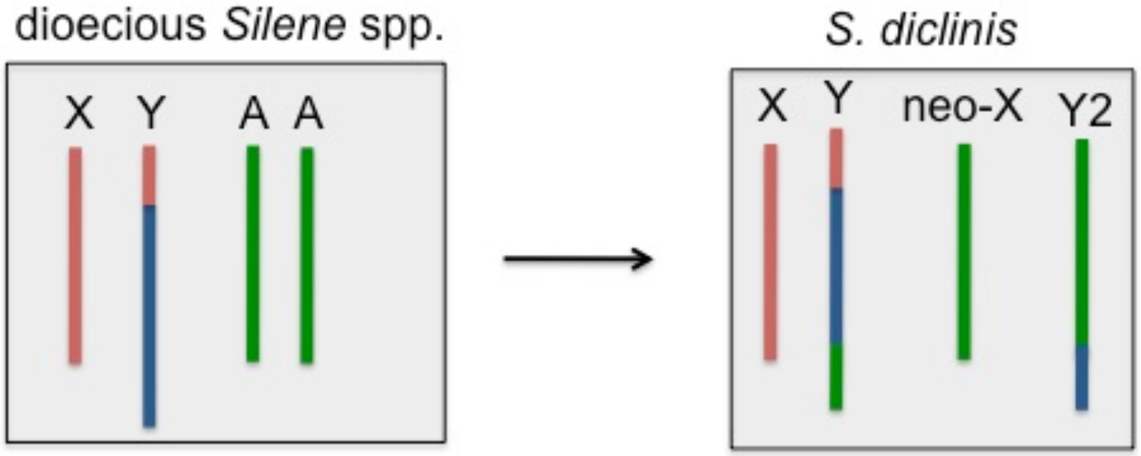
Table 1. Primers used for FISH genic probes.

Gene	*Direction*	*Primer Sequence*
SlY1	Forward	AAG CTC ACA ATG CTG ATC TTC
	Reverse	CAT ATA CTC CCT CAA TTC ACT TGG ACA
SlY4	Forward	TAG ACA AGG GCT GGG CTA CA
	Reverse	AAA ACC CAC CAT CAG TTG GA



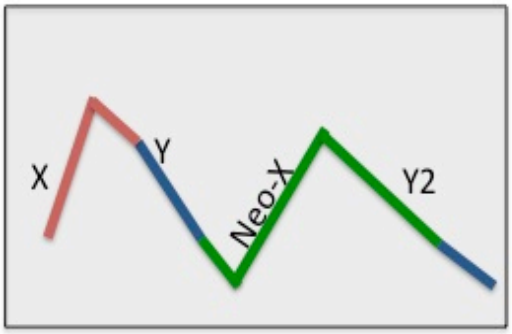


Figure 1. Above: A reciprocal translocation event between the Y chromosome and an autosome resulting in a second Y chromosome. The corresponding autosome is referred to as the neo-X chromosme. Left: A depiction of how the sex-chromosomes and neo-sex chromosomes are arranged as a quadrivalent chain.

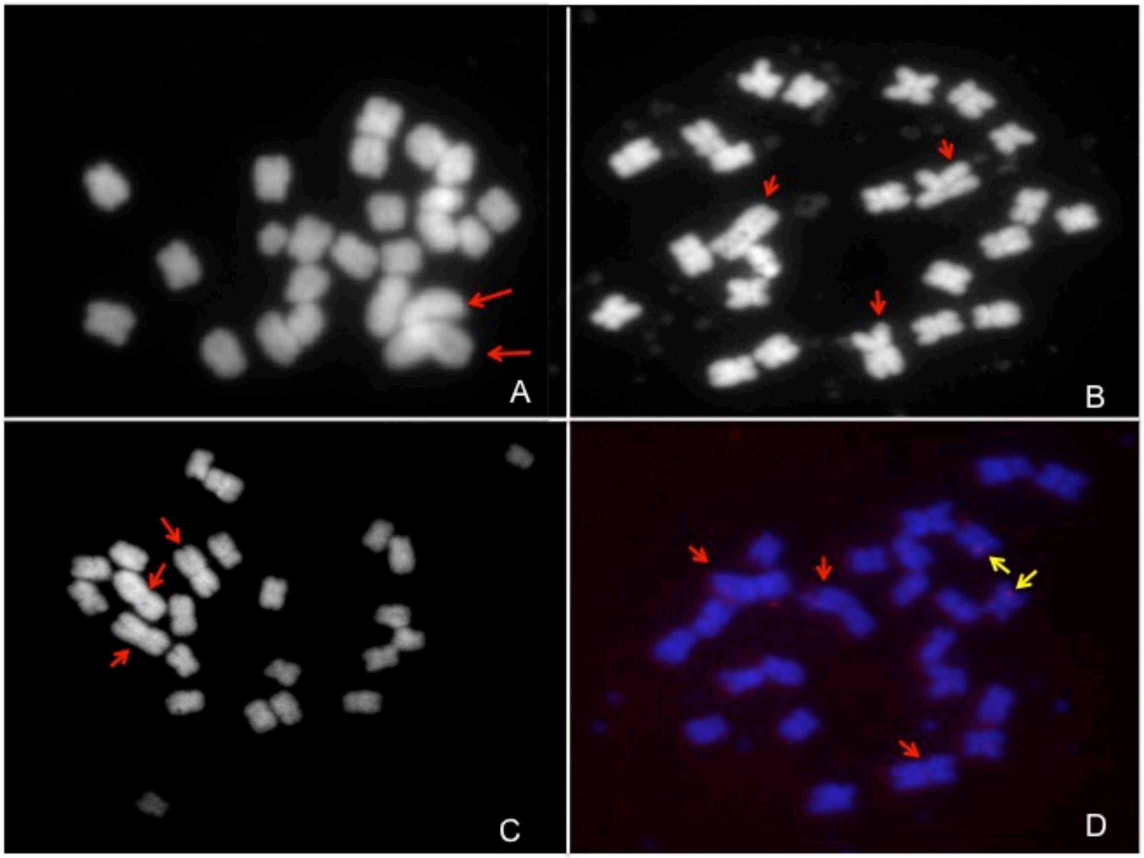


Figure 2. Karyotypes from root tip squashes. A) Pure *S. latifolia* male. Red arrows indicate the X and Y chromosome. B and C) *S. latifolia* by *S. diclinis* F1 hybrids. Red arrows indicate the X and Y chromosome as well as a third large chromosome, which may be the Y2. D) Hybridized F1 hybrid with red arrows indicating the three largest chromosomes. The yellow arrows indicate regions labeled by the SlY1 genic probe, though staining appears to be non-specific to the targeted regions.

CHAPTER 5

REPRODUCTIVE ISOLATING BARRIERS AMONG CLOSELY RELATED DIOECIOUS *SILENE*: CONCLUSIONS AND FUTURE DIRECTIONS.

Pre- and post-zygotic isolating barriers are important for maintaining species boundaries, especially among closely related organisms that occur in sympatry (Coyne and Orr 2004). However, there are still many questions about how new species are formed and how species boundaries are maintained. Even when the isolating barriers preventing gene flow in the present are clear, it is rarely possible to know the order in which they arose, and consequently which one or ones played the most important role in the speciation process. Indirect evidence gleaned from studies of reproductive isolation suggests that in plants, pre-pollination barriers arise first (Rieseberg and Willis 2007). However, even if it is possible to identify the species boundaries that are currently acting to prevent gene flow, it may still be difficult to know how each isolating barrier ranks in the order of importance. While these challenges may be daunting, it is also for these reasons that speciation has fascinated biologists since Darwin first put forward his theories regarding the origin of species, that "mystery of mysteries" (Darwin 1859). Since then, numerous studies regarding the ways that species boundaries are maintained have accumulated and there are stunning examples in the literature illuminating the mechanisms of speciation, such as Darwin's finches (Grant and Grant 1976) and cichlid fish (reviewed in Kornfield and Smith 2000), as well model systems that have provided invaluable tools such as *Drosophila* and *Helianthus* (Heiser 1973, Rieseberg 2001, Orr 2001, Coyne and Orr 2004, Orr et al. 2007, Rieseberg and Willis 2007). But there are also numerous examples of organisms that are less well suited to speciation studies, and yet have a great deal to teach us about the generality, diversity, and complexity of how new species can emerge and how species boundaries are maintained. The goal of my dissertation was to examine some of the reproductive isolating barriers in three closely related dioecious species of *Silene* with heteromorphic sex chromosomes. *Silene latifolia* has been the subject of numerous studies in the areas of ecology, evolution, and genetics (Bernasconi et al. 2009). It has become a model organism for studying life-history evolution, sex-chromosome evolution, and plant-parasite interactions. By utilizing the range of ecological, genetic, and cytogenetic tools available for *S. latifolia*, we gained new insights into some of the mechanisms important for maintaining species boundaries among *S. latifolia* and its sister species, *S. dioica* and *S. diclinis*.

In chapter 2, we examined pollinator mediated selection on floral traits in two closely related species, *S. latifolia* and *S. diclinis*, which occur sympatrically in a small region of Eastern Spain. Using an independently segregating population of artificially generated hybrids between the two species, we examined how suites of floral traits influence pollinator visitation. Prior to conducting fieldwork, it was unclear how closely these two species occurred and consequently it was not certain that pollinator mediated selection would be an important barrier to gene flow at present. While the two species were reported to have overlapping ranges (Prentice 1976), the proximity of the two species population locations was not reported. However, we found that populations of the two species occur within less than 200 meters and well within the traveling distance of the nocturnal pollinator of *S. latifolia*, *Hadena bicruris*, and also likely within the flight distance of many bees and flies. Given that *S. latifolia* and *S. diclinis* readily hybridize in greenhouse studies, the absence of phenotypically detectable hybrids in the field is somewhat surprising. While the main focus of our study was to determine the floral traits that influence pollinator attraction, seed set, and predation, we were also able to make some preliminary conclusions about the importance of pollinators for preventing gene flow in this system. In pure species used in observation arrays, we never observed moths visiting *S. diclinis* individuals. This suggests that there are floral traits that are unappealing to *Hadena*. It is possible that floral scent, nectar volume, nectar concentration, or other traits not measured in our study are important cues to nocturnal visitors. In our study, we were able to conclude that flower height influenced pollinator attraction and visitation, while flower size improved seed set, but also increased predation. This suggests that plant vegetative structure (*S. diclinis* are much shorter and flat against the ground), may be under disruptive selection by pollinators, and balancing selection may be constraining the evolution of flower size. Future studies should also document the frequency of visitation to natural populations by both diurnal and nocturnal populations. Furthermore, genetic studies should look for signs of introgression between the two species within their range. Although we did not observe hybrids of *S. latifolia* and *S. diclinis* in the field, it is known that in areas where *S. latifolia* occurs sympatrically with another sister species, *S. dioica*, widespread genome admixture has not been observed, but evidence for introgression between the two species has been documented (Minder 2007, Karrenberg and Favre 2008). Additionally, in collaboration with other members of the Delph lab, we are testing whether any local adaptation has occurred by conducting cross-pollination studies between sympatric populations of *S. diclinis* and *S. latifolia* and between *S. diclinis*, and allopatric populations of *S. latifolia*. Preliminary results have suggested that there may be reduced seed set among sympatric crosses, suggesting that genetic barriers reinforcing species boundaries in sympatry may have evolved. Finally, it is important to note that *S. diclinis* is a rare and endangered endemic species. Habitat loss, as well as encroaching development, continue to put this species at risk of extinction. Studies involving this species and highlighting its unique biology and ecology should be used to raise awareness about *S. diclinis* and inform its conservation.

In chapter three, we investigated whether Haldane's rule extends to plants with heteromorphic sex chromosomes. Haldane's rule has been demonstrated in numerous animal taxa including species with heterogametic males (XY) and in species with heterogametic females

(ZW). Because few plant species have sex chromosomes, and even fewer have heteromorphic sex chromosomes, *S. latifolia*, *S. dioica*, and *S. diclinis* are uniquely suited to studying the causes and consequences of Haldane's rule in plants. We found that F1 hybrids among *S. latifolia*, *S. diclinis*, and *S. dioica* suffer greater inviability and sterility relative to pure species crosses. Many of the limitations for understanding the causes of Haldane's rule in animal studies can be overcome in plants where crosses can be repeated among species and generations. Future work on Haldane's rule should consider a wide range of taxa and continue to investigate the scope of Haldane's rule. We have demonstrated that even in species with relatively young sex chromosomes, hybrid inviability and sterility are present in the heterogametic sex. Studies that examine hybrid inviability and sterility in plant species without heteromorphic sex chromosomes, or with heterogametic females, will be important for shedding light on the generality of Haldane's rule for plant speciation.

Chromosomal rearrangements have been implicated in speciation events across a wide range of plant taxa (Rieseberg 2001). Hybridization among species with different karyotypes are thought to suffer from reduced fertility and viability (Rieseberg 2001). In chapter 4, we examined the karyotypes of hybrids where one species, *S. latifolia*, is heteromorphic with 2n=22 + XY, and the other species, *S. diclinis*, has undergone a reciprocal translocation event resulting in a set of neo-sex chromosomes which form a stably inherited quadrivalent chain at meiosis (2n=20 + XY + neo-X and Y2) (Howell et al. 2009). Chromosomal rearrangements among autosomes are not uncommon in plants, but the importance of neo-sex chromosomes in speciation events is relatively unknown, especially because sex chromosomes are rare in plants. To our knowledge, the only example of a study of hybridization between individuals with and without neo-sex chromosomes comes from threespine sticklebacks (Kitano et al. 2009). In hybrid crosses, male threespine sticklebacks suffer from reduced mating success and there is evidence for an asymmetric reduction in fertility (Kitano et al. 2009). In chapter 4, we found that in F1 hybrids between *S. latifolia* and *S. diclinis* in which *S. diclinis* is the paternal parent, the two Y chromosomes are inherited by male offspring. We also observed in chapter 3 that hybrids between *S. latifolia* and *S. diclinis* suffer from reduced viability and fertility. While we are unable to draw conclusions about the direct effects of neo-sex chromosomes on speciation in this system, we think there is a great deal of potential for future studies. For example, we would like to use fluorescent in situ hybridization (FISH) to definitively identify the X chromosome, the Y chromosome, and the Y2 chromosome. Other laboratories have had success with genic probes in both *S. latifolia* and *S. diclinis* and we are close to being able to use these tools in hybrids. Additionally, other groups (Markova et al. 2006) have successfully used genomic *in Situ* hybridization (GISH) to differentiate between the chromosomes of two species in hybrids of *S. latifolia* and *S. viscose*. Successful development of whole-genome probes between *S. latifolia* and *S. diclinis* could aid in understanding whether these two species can recombine in certain regions of their genome. Evidence for reduced recombination associated with the neo-sex chromosomes would suggest that these chromosomal rearrangements effectively reduce or eliminate gene flow. Thus, while reduced viability and sterility in hybrids of *S. latifolia* and *S. diclinis* demonstrates that post-zygotic barriers are at work in this system, we have not observed any karyotypic abnormalities that would suggest that the neo-sex chromosomes affect viability. However, more work looking at specific genomic regions will provide insight into the importance of neo-sex chromosomes in this system.

It has been well documented in plants that there are many pre- and post-zygotic barriers that may contribute to reproductive isolation among closely related species (reviewed in Coyne and Orr 2004, Rieseberg 2007, Kay and Sargent 2009). The research presented in this dissertation focused on three specific reproductive isolating barriers. Based on our findings, both pre-and post-zygotic isolating barriers are contributing to maintaining species boundaries between closely related dioecious species of *Silene* with heteromorphic sex chromosomes. Specifically, we have shown that pollinator-mediated selection contributes to preventing gene flow between species in sympatry and that some floral traits may be more important than others. We have documented the first case of Haldane's rule in plants. Finally, we demonstrated that crosses between species where one has neo-sex chromosomes and the other does not, viable hybrids are still possible, though the effects of neo-sex chromosomes on hybrid fitness should be examined more closely. Taken together, these studies highlight the complex nature of pre- and post-zygotic isolating barriers in closely related species and underscore both the complex and fascinating aspects of plant speciation.

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Publications

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Talks

Brothers, A. and Delph, L.F., Nov 23, 2009. Reproductive isolating barriers in *Silene*., Invited seminar, Kent State University, Kent, OH

Brothers, A. and Delph, L.F., Oct 6, 2009. Pre-and Post-zygotic isolating barriers in *Silene*. Indiana University, Dept. of Biology Brown Bag seminar, Bloomington, IN

Brothers, A. and Weekley, C.W., April 3, 2004. Failure of reproductive assurance in the chasmogamous flowers of Polygala lewtonii (Polygalaceae), an endangered sandhill herb. Plant Biologists of South Florida, Big Cypress National Preserve, FL

Posters

Brothers, A. Nov 14, 2009. The generality of Haldane's rule is extended to plants with sex chromosomes. NSF- IGERT symposium, Indiana University.

Brothers, A. June 21, 2008. The role of pollinator-mediated selection in trait divergence between two species of Silene. Evolution Meeting, University of Minnesota

Brothers, A. March 10, 2007. Integrating floral scent into pollination syndromes. Midwest Ecology and Evolution. Kent State University, Kent, Ohio

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