3-2012

ASSESSING PATTERNS OF HYBRIDIZATION AND MULTIPLE MECHANISMS OF REPRODUCTIVE ISOLATION BETWEEN *ERYTHRONIUM ALBIDUM* AND ITS CONGENER *E. MESOCHOREUM*

Kathy Roccaforte
University of Nebraska-Lincoln, rocca@huskers.unl.edu

ASSESSING PATTERNS OF HYBRIDIZATION AND MULTIPLE MECHANISMS OF REPRODUCTIVE ISOLATION BETWEEN *ERYTHRONIUM ALBIDUM* AND ITS CONGENER *E. MESOCHOREUM* (LILIACEAE).

By

Kathy Roccaforte

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Biological Sciences

Under the Supervision of Professors Sabrina E. Russo and Diana Pilson

Lincoln, Nebraska

March, 2012

ASSESSING PATTERNS OF HYBRIDIZATION AND MULTIPLE MECHANISMS OF REPRODUCTIVE ISOLATION BETWEEN *ERYTHRONIUM ALBIDUM* AND ITS CONGENER *E. MESOCHOREUM* (LILIACEAE)

Kathy Roccaforte, M.S.

University of Nebraska, 2012

Advisors: Sabrina E. Russo and Diana Pilson

Although there are approximately 250,000 extant angiosperm species, we still have much to learn about the speciation process, including the ways in which species boundaries are maintained among closely related taxa. Species are formed when populations become reproductively isolated from each other via genetic and morphological barriers that act before hybrid formation (prezygotic) or after hybrids are formed (postzygotic).

Erythronium albidum Nutt. and *Erythronium mesochoreum* Knerr (Liliaceae) differ in ploidy, are likely sister species, and have been reported to hybridize, making them well-suited for assessing the strength of multiple reproductive barriers. First, I assessed the frequency of hybrid occurrence at four contact zones throughout the U.S. Midwest. Hybrids were identified based on genome size using flow cytometry. I found that hybrids occurred infrequently, indicating that reproductive isolation between the study species is strong.

Next, I assessed the contributions of numerous pre- and postzygotic barriers to species boundary maintenance between *E. albidum* and *E. mesochoreum.* Using herbarium records from Midwestern states and study plots in eastern Nebraska, I found that flowering phenology for each species differs significantly on a broad

geographic scale but can overlap substantially on local scales. This indicates that flowering asynchrony is not a consistently strong reproductive barrier. Further, by capturing insects visiting *E. albidum* and *E. mesochoreum* flowers, I found that the plants' pollinator assemblages are significantly non-overlapping, which may serve as a strong reproductive barrier by severely limiting interspecific pollen transfer. Finally, in a hand-pollination experiment, I found that hybrid seed set was significantly lower than conspecific seed set.

Overall, these studies show that multiple reproductive barriers contribute to the maintenance of species boundaries between *E. albidum* and *E. mesochoreum*. However, these barriers varied in strength. Though many previous studies have emphasized the role of individual reproductive barriers for species formation and perpetuation, my results highlight the importance of considering the role of multiple barriers in species boundary maintenance among plants.

THESIS DEDICATION

This thesis is dedicated to the memory of my grandmother, Mary Roccaforte, who always emphasized to her grandchildren the importance of lifelong learning.

AUTHOR'S ACKNOWLEDGEMENTS

First, I would like to thank my advisors, Drs. Sabrina E. Russo and Diana Pilson. You have both provided invaluable guidance to me during the last three years. Under your tutelage, I have learned quite a bit about ecology and evolution. More importantly, you have helped me to learn *how to be a scientist*, and those are skills that I will always take with me. Thank you for being my mentors.

I also owe a debt of gratitude to my other committee members, Drs. Robert Kaul and Stacey Smith. Dr. Kaul first planted the "*Erythronium* seed" in my mind, and Dr. Smith was willing to start providing guidance as a new faculty and committee member before she even set foot in Lincoln. Thank you both for being so generous with your time and expertise.

Russo lab members, past and present, have also provided critical feedback, friendship, an introduction to Thai food, and comic relief (not all at the same time). Thank you especially to Katie Heineman, Jocelyn Olney, Amy Kochsiek, Noelle Beckman, Melissa Whitman, and Shivani Jadeja.

Danielle Tufts and Heidi Bulfer, I owe you both debts of gratitude for being a great source of friendship over the past three years. Whether we were collecting insects together or dressing up like them for Halloween, we have always managed to have a great time. All for one, and one for all!

The numerous field-based projects associated with my research would not have been feasible without the assistance of a large number of people. Thank you to the undergraduate "Trout Lily Trackers", especially Jose Morales and Amber Kula,

for all of your help with fieldwork and data entry. Another round of thanks goes out to my family members and friends—Bill, Jane, and Mike Roccaforte, Stephanie Miske, and Emily and Matt Kasprzak—for providing love and support, as well as for willingly spending several hours hunched over tiny plants these past two field seasons. My fieldwork would have been infinitely less interesting and enjoyable without the assistance of Glenn Pollock, as well. Thank you, Glenn, for all of your help and encouragement, and for regaling me with stories of the history of the Great Plains. You have taught me so much, and I am very appreciative.

Dr. Charles Kuszynski, Dr. Eric Baack, Danielle Shea, and Zhi Hong Gill provided critical assistance with flow cytometry. Thank you, and sorry for constantly clogging up the cytometer with plant tissue!

I would also like to thank several researchers associated with the USDA-ARS Systematic Entomology Laboratory for providing insect identifications— Sam Droege (Hymenoptera identifications), Biologist, USGS Patuxent Wildlife Research Center; Neal L. Evenhuis (Diptera: Bombyliidae identifications), SEL Cooperating Scientist, B.P. Bishop Museum, Honolulu, HI; Norman E. Woodley (Diptera: Anthomyiidae identification), Research Entomologist, SEL.

Last but not least, I would like to thank Patrick Denning for his love and support during these busy, stressful months. I owe you a large five-mushroom pizza.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION: USING SISTER SPECIES IN THE GENUS *ERYTHRONIUM* (LILIACEAE) TO STUDY SPECIATION AND HYBRIDIZATION

INTRODUCTION

Investigations of the processes that govern speciation, hybridization, and the maintenance of species boundaries trace their origins to the foundation of modern biology (Figure 1.1), and these studies are integral to the fields of ecology and evolutionary biology. Recent decades have seen a surge of speciation research as interest in the topic has been rekindled (Sobel *et al.* 2010). However, despite the considerable progress that has been made, we still have much to learn regarding both how new species are formed and how extant species remain genetically and morphologically distinct from one another. The overarching goals of my master's thesis research are to investigate patterns of natural hybridization and assess the contribution that multiple isolating barriers play in the maintenance of species boundaries between two closely related trout lilies (*Erythronium* spp.) that differ in ploidy. This research will further our knowledge of the ways in which reproductive isolation is maintained among plant taxa, and it will contribute to our understanding of plant speciation, especially with regard to diploid-polyploid species pairs.

PART I: SPECIES CONCEPTS AND POLYPLOID SPECIATION

Species Concepts

One of the fundamental challenges in the field of speciation research remains determining what, precisely, constitutes a species. While it is more or less intuitive for scientists to informally understand species as "definable biological groups of distinct lineage and with potentially independent futures (Hendry 2009)", there remains a vigorous debate among evolutionists as to what, biologically, comprises a species as well as what delineates distinct species from one another (Coyne & Orr 2004). This "species problem" troubled Charles Darwin as well. In *On the Origin of Species*, Darwin had difficulty with the concept of species, stating:

> In short, we shall have to treat species in the same manner as those naturalists treat genera, who admit that genera are merely artificial combinations made for convenience. This may not be a cheering prospect; but we shall at least be free from the vain search for the undiscovered and undiscoverable essence of the term species (Darwin 1859).

Needless to say that despite Darwin's reservations, many biologists have indeed attempted to define, biologically, what constitutes a species. These conceptual frameworks are referred to as "species concepts".

Perhaps the most well-known and widely used species concept is the biological species concept (BSC). The BSC states that species are groups of interbreeding organisms that are *reproductively isolated* from other such groups (Dobzhansky 1935, Mayr 1942). Reproductive isolation occurs when one or more

traits, termed reproductive barriers, limits or prevents the formation of fertile hybrids between members of two taxa. Reproductive barriers are generally divided into two categories— prezygotic and postzygotic. Prezygotic barriers occur prior to hybrid fertilization, and include myriad isolating mechanisms such as habitat-based spatial differentiation, interspecific differences in courtship and mating behavior, and physiological (often called "intrinsic") barriers that prevent the fertilization of hybrid embryos. Postzygotic barriers occur after hybrid fertilization and include hybrid inviability and sterility (Coyne & Orr 2004). Delineating species under the framework of the BSC involves assessing, in the field or laboratory, whether taxa are reproductively isolated from one another.

Despite the usefulness and popularity of the BSC, many evolutionists have remained troubled by some of its limitations and have proposed alternative species concepts. Critics of the BSC generally point to a number of challenges, three of the most troubling of which are presented below.

First, under the framework of the BSC, it is difficult to determine whether populations existing in allopatry (*i.e.* populations that do not overlap spatially) can be considered distinct species. It is often unclear whether allopatric populations are capable of interbreeding unless they are purposely brought into close contact in the field or laboratory, which is typically logistically difficult and may disrupt traits that lead to reproductive isolation in the natural habitats of the study taxa (Hendry 2009).

Second, species delimitation under the framework of the BSC requires reproductive isolation, but hybridization is common across many taxa in nature.

Estimates of the frequency of hybridization vary widely, and some reports have indicated that up to 25% of species within various taxonomic groups can successfully interbreed with other species (Schwenk *et al.* 2008). This has led biologists to question the extent to which taxa can hybridize but still be considered distinct species (Mishler & Donoghue 1982). It is important to note that many modern proponents of the BSC do not require complete reproductive isolation to occur between taxa for them to be recognized as distinct species (Coyne & Orr 2004).

Finally, species delineations based on the BSC are impossible to make for organisms that do not reproduce sexually or that are extinct and require classification based solely on fossil evidence (Hendry 2009). There are a wealth of organisms, including numerous prokaryotes and other microorganisms, that do not exhibit sexual reproduction and biparental inheritance. For scientists studying speciation and diversification in these taxa, the BSC is likely not a useable framework for species delimitation (Coyne & Orr 2004).

The challenges posed by the BSC have lead to the development of a wealth of additional species concepts that attempt to address these problems (Table 1.1).

However, despite the existence of numerous alternative species concepts, the BSC directly addresses what many evolutionists consider the most salient question in the field of speciation (Coyne & Orr 2004)—how do co-occurring taxa remain genetically distinct in the face of hybridization and gene flow? Regardless of the specific mechanisms underlying divergence (*i.e.* genetic drift in allopatry, disruptive selection in sympatry), speciation occurs as a consequence of the formation of

reproductive barriers, and defining species as groups that are reproductively isolated from one another links the observable phenomena (the presence of genetically and/or morphologically distinct groups) with the process (reproductive isolation) that produces them. Because of its focus on reproductive isolating mechanisms, as opposed to other features that can define species (Table 1.1), the research presented in this thesis has been formulated and carried out under the conceptual framework of the BSC.

The Origin and Maintenance of Polyploid Species

Evolutionists have long recognized the potential importance of polyploidy and polyploid speciation in the evolutionary history of angiosperms. Polyploid species have ≥ 3 genomic copies, and polyploid speciation occurs when speciation is accompanied by an increase in ploidy. Polyploidy has occurred numerous times throughout the evolutionary history of flowering plants (Soltis *et al.* 2003). Estimates of the frequency of polyploidy vary, but some studies indicate that up to 80% of angiosperms have experienced genome duplication at some point in their evolutionary history (Soltis & Soltis 2009, Pires & Gaeta 2011). Even ancient genome duplication (paleopolyploidy) has left a genetic signature on angiosperm genomes across many taxa (Blanc *et al.* 2003, Wang *et al.* 2005, Schmutz *et al.* 2010), and duplicated genes resulting from paleopolyploidy may play an important role in critical cellular functions such as signal transduction and transcriptional regulation (Arrigo & Barker in press). Further, it is estimated that approximately 15% of speciation events are accompanied by a change in ploidy (Wood *et al.* 2009).

However, despite the evidence of widespread polyploidy in the evolutionary history of angiosperms, there has been much recent debate regarding the evolutionary implications of genome duplication. Recent research has indicated that polyploid lineages actually diversify at *lower* rates than diploid lineages and suffer from a higher extinction risk (Wood *et al.* 2009, Mayrose *et al.* 2011). There is still much uncertainty regarding both how paleo- and neopolyploidy have influenced the genetic architecture of angiosperms and why polyploidy does not appear to be a path to diversification within angiosperm lineages. This recent work highlights the importance of studying polyploid speciation to further our understanding of the ways in which polyploidy has affected the evolutionary trajectories of angiosperms.

Polyploid speciation is unique among speciation processes in that strong postzygotic isolation between polyploids and their diploid progenitors can occur virtually instantaneously via genetic incompatibilities associated with genome duplication (Husband 2004). Nearly complete reproductive isolation among plant species that differ in ploidy is commonly thought to be conferred by triploid block, a form of intrinsic postzygotic isolation wherein triploid hybrids are sterile or inviable as a result of numerous factors, including abnormal seed development and aneuploid gamete formation (Marks 1966, Coyne & Orr 2004). Aside from conferring strong reproductive isolation in and of itself, the production of low-fitness, triploid hybrids may reinforce incomplete species boundaries by creating a selection pressure for the evolution of prezygotic barriers that reduce the frequency of inter-cytotype hybridization.

Though triploid block has been observed in many plant taxa, its occurrence is not uniform across angiosperms (Ramsey & Schemske 1998). Additionally, mathematical models indicate that even low-fitness triploids may *promote* polyploid formation by serving as a "bridge" for the creation of new polyploid individuals (Felber & Bever 1997, Husband 2004). Further, although studies assessing the strength of multiple isolating mechanisms between taxa that differ in ploidy are few, additional barriers have been shown to play a role in reproductive isolation between diploids and polyploids (Petit *et al.* 1999, Husband & Schemske 2000, Glennon *et al.* in press).

Overall, our understanding of the mechanisms underlying the origin and maintenance of species boundaries between diploid-polyploid species pairs and cytotypes remains rudimentary (Husband & Sabara 2003). It is also clear that closely related diploid-polyploid species pairs, as well as plant species with multiple cytotypes, serve as excellent natural systems in which to address these critical questions in the field of plant speciation. In addition, many recent studies have focused intensely on evaluating the role that single reproductive barriers play in plant speciation (Rieseberg *et al.* 1995, Bradshaw & Schemske 2003, Hentrich *et al.* 2010). Relatively few studies, however, have investigated the contributions of multiple barriers to reproductive isolation between closely related plant taxa (Rieseberg & Willis 2007, Widmer *et al*. 2009, but see Ramsey *et al.* 2003, Kay 2006, Lowry *et al.* 2008). My research addresses these gaps in our understanding of plant speciation for which additional studies are vitally needed by utilizing a diploid-

tetraploid plant species pair to evaluate the contribution of multiple reproductive barriers to the maintenance of species boundaries between closely related taxa.

Erythronium albidum Nutt. and *Erythronium mesochoreum* Knerr (Liliaceae) are ideally suited to studies of hybridization and reproductive isolation among diploid-polyploid species pairs for three reasons, which I will introduce here but elaborate upon in the next section. First, *E. albidum* and *E. mesochoreum* differ in ploidy but share the same base chromosome number and are likely sister species (Allen *et al.* 2003). The difference in genome copy number between the two species allows for rapid, accurate detection of hybrids using flow cytometry. Second, *E. albidum* and *E. mesochoreum* are sympatric, and populations of the two species overlap on local scales. This makes them ideal for testing reproductive barriers in sympatry. Third, hybridization between *E. albidum* and *E. mesochoreum* has been reported in eastern Nebraska (R.B. Kaul, unpublished data), which suggests that species barriers between *E. albidum* and *E. mesochoreum* may be incomplete and of relatively recent origin.

PART II: *ERYTHRONIUM* BIOGEOGRAPHY, MORPHOLOGY AND NATURAL HISTORY

Erythronium Morphology and Geographic Distribution

Members of the genus *Erythronium* (Liliaceae) are commonly called trout lilies or dog-tooth violets. They are geophytic, bulbous perennials bearing one to three often mottled, lanceolate to ovate leaves, and one to approximately 20 small but showy flowers colored white, yellow, cream or violet (Mathew 1992). *Erythronium* has a broad geographic distribution and is comprised three clades—

the Eurasian clade (four species), the eastern North American clade (six species), and the western North American clade (about 17 species) (Allen 2008; Figure 1.2). The majority of *Erythronium* species are diploid, although at least five species are tetraploid (Allen 2008). Members of the Eurasian and western North American clades, as well as three of the six eastern North American *Erythronium* species, bear a base chromosome number of x =12. The remaining three species, all occurring in the eastern North American clade, have a base chromosome number of x = 11 (Allen *et al.* 2003; see below).

Erythronium albidum and Erythronium mesochoreum

Morphology, Geographic Distribution, and Life History

E. albidum and *E. mesochoreum* belong to *Erythronium*'s eastern North American clade. Within eastern North America, *E. albidum* has a broad geographic range, extending from the Midwest to the east coast and from southern U.S. states north into Ontario. *E. mesochoreum*'s range is much narrower, as it is restricted to central U.S. states (Figure 1.3; Allen & Robertson 2002). *E. albidum* and *E. mesochoreum* tend to inhabit different types of habitat—*E. albidum* is typically found in mesic woodlands, whereas *E. mesochoreum* is generally restricted to tallgrass prairies (Churchill 1986, Kaul 1989). However, populations of both species can abut at prairie-forest borders, and populations can intergrade widely in intermediate habitats such as savannahs (K. Roccaforte, personal observation).

Both species are perennial spring ephemerals, emerging in late March or early April and senescing by mid to late May. Flowering plants bear two to three leaves and single white flowers. Non-flowering plants, which are either non-

reproductive or vegetatively reproducing, bear one leaf. The seeds of *E. albidum* and *E. mesochoreum* have elaiosomes and are, in part, dispersed by ants (Churchill 1986; Figure 1.4).

E. albidum and *E. mesochoreum* are similar in appearance, but they can generally be distinguished from one another in sympatry based on several morphological characteristics (Figure 1.5). *E. albidum* has mottled leaves, whereas *E. mesochoreum*'s leaves are typically unmottled. In addition, the leaves of *E. albidum* are generally slightly folded, whereas *E. mesochoreum*'s leaves are conduplicate. Further, while both plants bear flowers with 6 tepals, *E. albidum*'s perianth is typically more reflexed than *E. mesochoreum*'s, and *E. albidum*'s fruits are held more erect (Knerr 1891, Churchill 1986, Kaul 1989). It becomes much more difficult to discern *E. albidum* from *E. mesochoreum* later in the spring, as *E. albidum*'s characteristic leaf mottling fades. However, I have noticed that, in eastern Nebraska, *E. mesochoreum* fruits dehisce earlier and that dehiscence occurs while the fruits remain attached to the scape, whereas *E. albidum*'s scapes senesce while the fruits are still intact (K. Roccaforte, personal observation).

E. albidum and *E. mesochoreum* also differ in their mode and frequency of asexual reproduction. *E. albidum* reproduces clonally by forming stolons that terminate in daughter bulbs (Muller 1979). Vegetative reproduction is much more common for *E. albidum* than sexual reproduction, and it allows this species to form large, dense colonies consisting primarily of single leaved, non-flowering plants (Muller 1979, Banks 1980). In contrast, *E. mesochoreum* reproduces asexually via bulb offshoots (Kaul 1989). Plants within *E. mesochoreum* populations tend to be

less densely packed (Kaul 1989), although populations can still consist of several thousand individuals (K. Roccaforte, personal observation).

Detailed studies of *E. albidum* and *E. mesochoreum*'s pollinator assemblages are lacking, but two insect species that play a large role in the pollination of these species have been identified. *E. mesochoreum* is primarily pollinated by the solitary bee *Andrena erythronii* (Andrenidae), and this insect species is likely dependent on *E. mesochoreum* as its primary source of pollen (Michener & Rettenmeyer 1956). *E. albidum*'s chief pollinator is also a solitary Andrenid bee—*Andrena carlini* (Banks 1980). *Andrena erythronii* and *Andrena carlini* forage for pollen and nectar in *Erythronium* flowers, and the pollen that the females collect is used to provision eggs laid in underground nests (Michener & Rettenmeyer 1956, Schrader & LaBerge 1978, Banks 1980).

Additional insect visitors to *E. mesochoreum* include various *Apis* species (Apidae) (Michener & Rettenmeyer 1956). Schemske *et al.* (1978) found that, while the most abundant floral visitor to *E. albidum* in an eastern Illinois woodland was *Andrena carlini*, *E. albidum* flowers were also visited less frequently by *Andrena masonii, Andrena erythronii, Andrena forbesii, Andrena erigeniae,* and *Apis mellifera*.

Chromosome Count, Ploidy, and Phylogenetic Relationship

Even though these species differ in several morphological traits, it can be difficult to discern *E. albidum* from *E. mesochoreum* in sympatry based on morphology alone (Ireland 1957). However, the two species can be readily distinguished based on genome copy number. Though they share a base

chromosome number of x = 11, *E. mesochoreum* is diploid (2n = 2x = 22) and *E. albidum* is tetraploid (2n = 4x = 44; Robertson 1966).

In addition, several lines of evidence strongly suggest that *E. albidum* and *E. mesochoreum* are sister species. Maximum parsimony cladograms based on sequence data from the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA genes identified a strongly supported clade consisting of *E. albidum*, *E. mesochoreum* and another eastern North American species, *E. umbilicatum* (Allen *et al.* 2003). Unfortunately ITS sequence data was unable to resolve the phylogenetic relationship among these three species. Allen *et al.*'s (2003) two maximum parsimony trees differed in their arrangement, placing either *E. albidum* or *E. umbilicatum* as sister to the other two taxa. However, karyotype data bolsters the argument that *E. albidum* and *E. mesochoreum* are, in fact, sister species. As stated above, both *E. albidum* and *E. mesochoreum* share the base chromosome number x = 11. Almost all other members of the genus *Erythronium*, including *E. umbilicatum*, have a base chromosome number of x =12 (the sole exception is *E. propullans,* which is likely a clonal derivative of *E. albidum* and is endemic to two counties in southeastern Minnesota; Banks 1980, Pleasants & Wendel 1989). This suggests that x = 11 is a derived base chromosome number. Thus, taking into account karyotype evidence, the most parsimonious relationship among these species places *E. albidum* and *E. mesochoreum* as sister species, with the x = 11 base chromosome number evolving only once (Allen *et al.* 2003).

Hybridization and the Application of Flow Cytometry to Detect Hybrids

One definitive record of hybridization between *E. albidum* and *E. mesochoreum* exists. An accession from the University of Nebraska-Lincoln's Charles E. Bessey Herbarium (NEB 306411; see Chapter 2), collected in 1995, consists of four plants that were identified as hybrids based on morphological characteristics assessed at the collection site, chromosome counts, and a genome size (assessed by flow cytometry) that was intermediate to those of the parental species (R.B. Kaul, unpublished data).

Because *E. mesochoreum* is diploid and *E. albidum* is tetraploid, it is reasonable to predict that hybrids are triploid and thus have a genome size intermediate to those of the parental species. These factors make *E. albidum* and *E. mesochoreum* ideal candidates for assessment of hybridization using flow cytometry. Flow cytometry is a cytological technique that allows one to rapidly quantify or qualify nuclear DNA content (Doležel *et al.* 2007). Over the past 25 years, flow cytometry has become an important tool in the analysis of ploidy variation among plants across a wide range of spatial scales (Burton & Husband 1999, Baack 2004, Trávníček *et al.* 2011). As such, flow cytometry is well-suited for investigating patterns of hybridization among taxa that differ in ploidy, especially when it is difficult to identify hybrids based on morphology (Suda *et al.* 2007).

CONCLUSIONS

Although investigations of the origin and maintenance of species boundaries are integral to the fields of evolutionary biology and ecology, few studies have

sought to identify multiple barriers that promote reproductive isolation between closely related plant taxa (Widmer *et al.* 2009), and this knowledge is certainly lacking among diploid-polyploid plant species pairs and cytotypes. *Erythronium albidum* and *E. mesochoreum* are an excellent study system in which questions regarding polyploid speciation and the role of multiple isolating mechanisms can be addressed, as the study taxa are likely sister species, they exist in sympatry, and they may be hybridizing naturally. The goals of this thesis are to assess the frequency of natural hybrid occurrence (Chapter 2), and to evaluate the contribution that multiple reproductive barriers play in the maintenance of species boundaries between *E. albidum* and *E. mesochoreum* across multiple Midwestern populations (Chapter 3; Figure 1.6). Ultimately, this research will contribute to our understanding of the mechanisms by which diploid-tetraploid plant species pairs maintain reproductive isolation in sympatry, which will further our understanding of plant speciation and hybridization.

TABLES

Table 1.1: The biological species concept and some alternative species concepts.

Species definitions are italicized, and relevant features are listed below.

Species Concepts, Including Alternatives to the Biological Species Concept
Biological Species Concept (BSC):
Species are groups of (potentially) interbreeding populations that are reproductively isolated from other such groups (Dobzhansky 1935, Mayr 1942)
• taxa can be delimited as distinct species, even under limited hybridization, using the BSC (Coyne & Orr 2004)
• it is difficult to delineate species for allopatric and asexual groups using the BSC
Genotypic Cluster Species Concept (GCSC):
Species are (genetically or morphologically) distinguishable groups of individuals that have few or no intermediates when in contact with one another (Mallet 1995)
• species can be diagnosed in the face of gene flow using the GCSC
• asexual taxa may be delineated as species under the GCSC
Evolutionary Species Concept (EvSC):
"An evolutionary species is a lineage (an ancestral-dependent sequence of populations) evolving separately from others and with its own unitary evolutionary role and tendencies" (Simpson 1961)
• asexual taxa may be delineated as species under the EvSC
Ecological Species Concept (EcSC):
"A species is a lineage (or closely related set of lineages) which occupies an adaptive zone minimally different from that of any other lineage in its range and which evolves separately from all lineages outside its range" (Van Valen 1976)
• asexual taxa may be delineated as species under the EcSC
• formulated in part specifically to delimit taxa that are sympatric yet exchange some genes (*e.g.* some North American *Quercus* spp.)
Genealogical Species Concept (GSC):
A species is a "basal group of organisms, all of whose genes coalesce more recently with each other than with those of any organisms outside the group" (Baum & Donoghue 1995)
• species are delineated based on historical relatedness (ancestry)

FIGURES

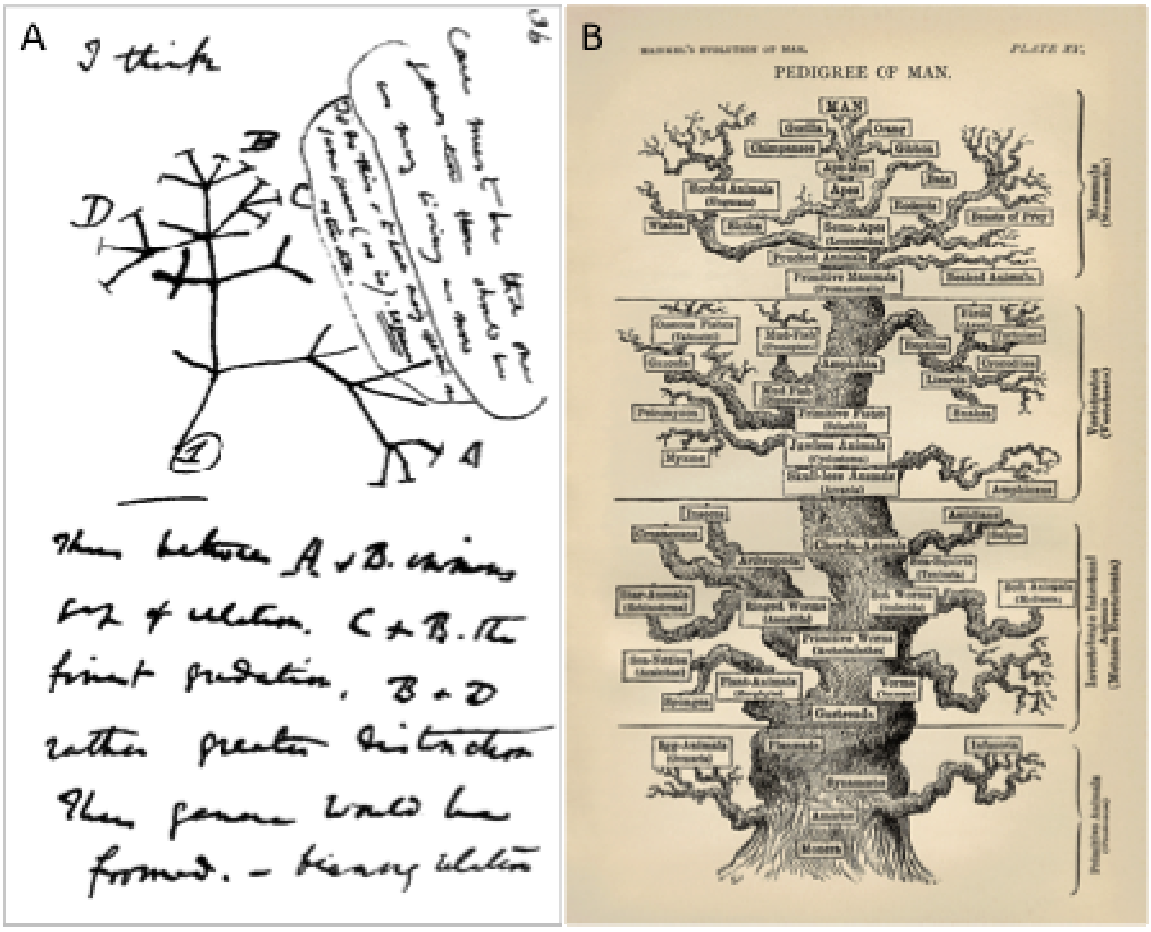

Figure 1.1: Illustrations of 19th century cladograms, which describe evolutionary relationships among species. **A.** Darwin's (1837) sketch of hypothetical relationships among species, from Notebook B; **B.** Illustration of the "Pedigree of Man" from Haeckel's (1879) *The Evolution of Man*. Both images are in the public domain of the United States (PD-U.S.), and were uploaded from Wikimedia Commons at http://commons.wikimedia.org.

Figure 1.2: Worldwide distribution of *Erythronium,* which consists of three clades—the eastern North American clade, the western North American clade, and the Eurasian clade (from Allen *et al.* 2003).

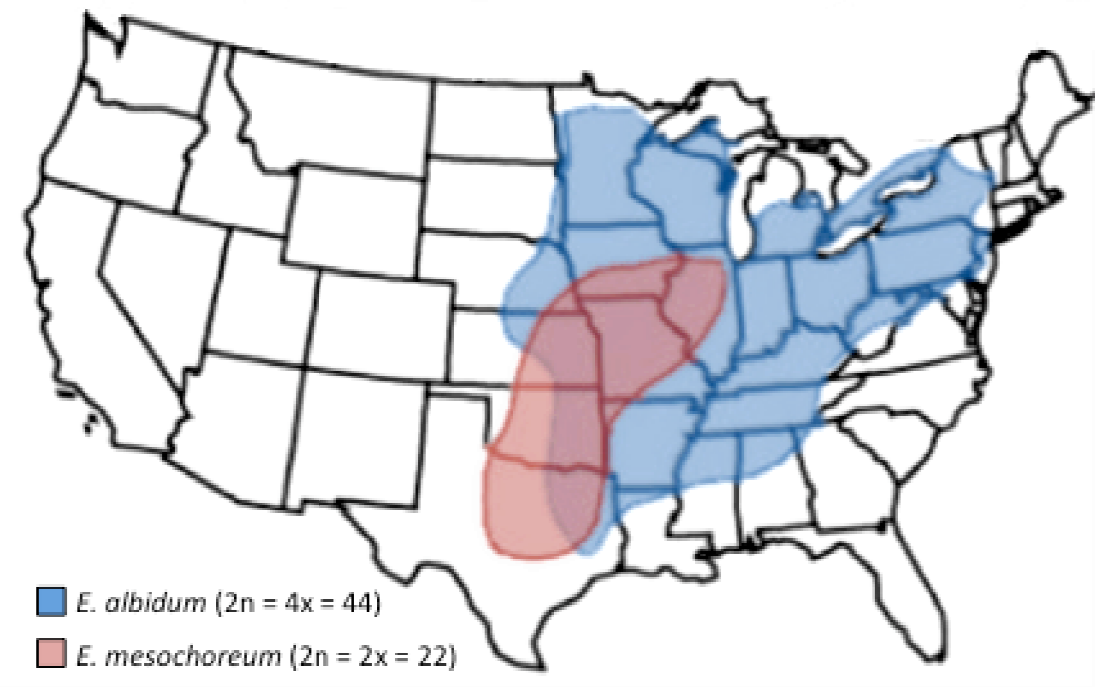

Figure 1.3: Geographic distributions of tetraploid *Erythronium albidum* and diploid *Erythronium mesochoreum* in the United States (adapted from Allen & Robertson 2002).

Figure 1.4: An ant dispersing an *E. albidum* seed at Pioneers Park Nature Center, Lincoln (Lancaster Co.), NE. Photo: K. Roccaforte.

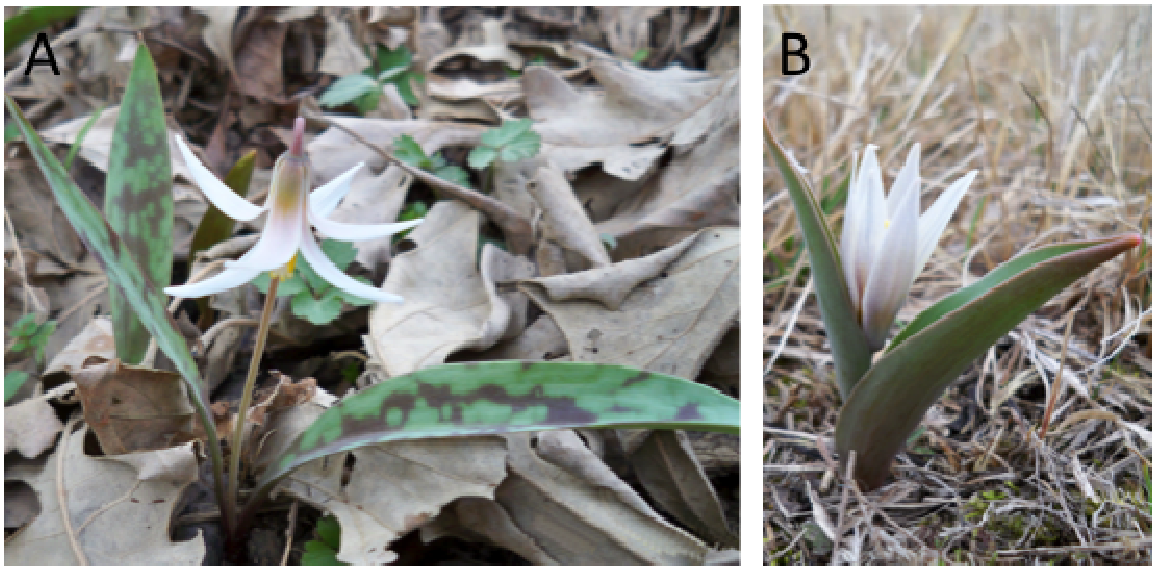

Figure 1.5: Distinguishing morphological characteristics of *Erythronium albidum* and *Erythronium mesochoreum*. **A.** *E. albidum* generally has slightly folded, heavily mottled leaves, and flowers with reflexed tepals. **B.** *E. mesochoreum* generally has conduplicate, unmottled leaves, and the flowers of *E. mesochoreum* typically have tepals that are less reflexed (Churchill 1986). Photos: K. Roccaforte.

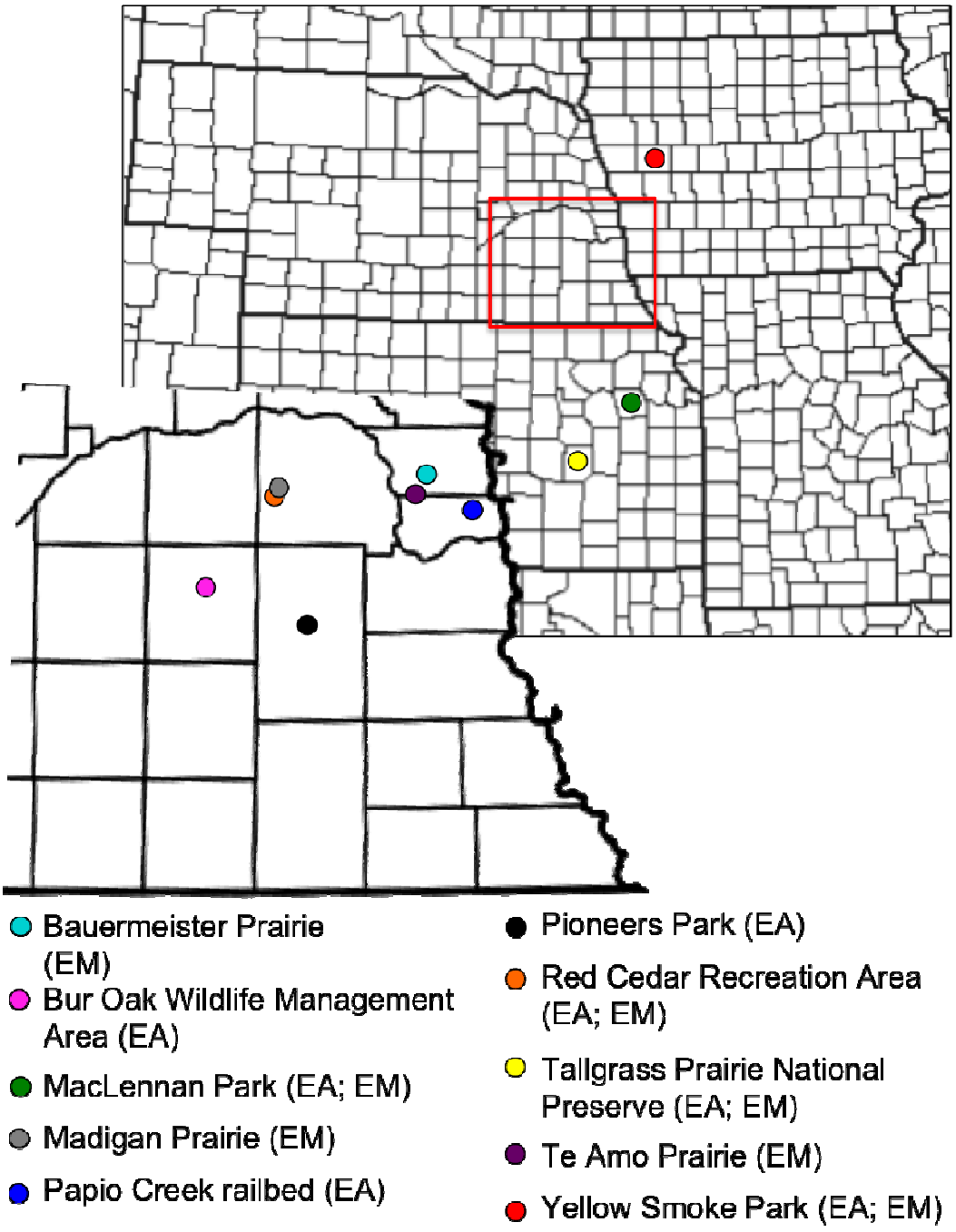

Figure 1.6: Map of Midwestern study sites utilized in studies of hybridization and reproductive isolation between *Erythronium albidum* (EA) and its congener *E.*

mesochoreum (EM). The inset map of eastern Nebraska (bottom left) is delineated by the red box.

REFERENCES

Allen, GA. 2008. The origins of polyploids in western North American fawn-lilies (*Erythronium*). Botany 86: 835-845.

Allen, GA. & Robertson, KR. 2002. Erythronium. pp. 153-164 *in* Flora of North America Editorial Committee (eds.) *Flora of North America, Vol. 26.* Oxford University Press, New York.

Allen, GA., Soltis, DE., & Soltis, PS. 2003. Phylogeny and biogeography of *Erythronium* (Liliaceae) inferred from chloroplast *matK* and nuclear rDNA ITS sequences. Systematic Botany 28(3): 512-523.

Arrigo, N. & Barker, MS. *In press*. Rarely successful polyploids and their legacy in plant genomes. Current Opinion in Plant Biology, *In press.*

Baack, EJ. 2004. Cytotype segregation on regional and microgeographic scales in snow buttercups (*Ranunculus adoneus*: Ranunculaceae). American Journal of Botany 91(11): 1783-1788.

Banks, JA. 1980. The reproductive biology of *Erythronium propullans* Gray and sympatric populations of *E. albidum* Nutt. (Liliaceae). Bulletin of the Torrey Botanical Club 107(2): 181-188.

Baum, DA. & Donoghue, MJ. 1995. Choosing among alternative "phylogenetic" species concepts. Systematic Botany 20(4): 560-573.

Blanc, G., Hokamp, K., & Wolfe, KH. 2003. A recent polyploidy superimposed on older large-scale duplications in the *Arabidopsis* genome. Genome Research 13: 137-144.

Bradshaw, HD. & Schemske, DW. 2003. Allele substitution at a flower color locus produces a pollinator shift in monkeyflowers. Nature 426: 176-178.

Burton, TL. & Husband, BC. 1999. Population cytotype structure in the polyploid *Galax urceolata* (Diapensiaceae). Heredity 82: 381-390.

Churchill, SP. 1986. Liliaceae. pp. 1241-1258 *in* Great Plains Flora Association (eds.) *Flora of the Great Plains*. University Press of Kansas, Lawrence, KS.

Coyne, JA. & Orr, HA. 2004. *Speciation*. Sinauer Associates, Sunderland, MA.

Darwin, CR. 1859. *On the Origin of Species or the Preservation of Favored Races in the Struggle for Life.* John Murray, London.

Dobzhansky, T. 1935. A critique of the species concept in biology. Philosophy of Science 2: 344-355.

Doležel, J., Greilhuber, J., & Suda, J. 2007. Estimation of nuclear DNA content in plants using flow cytometry. Nature Protocols 2(9): 2233-2244.

Felber, F. & Bever, JD. 1997. Effect of triploid fitness on the coexistence of diploids and tetraploids. Biological Journal of the Linnean Society 60: 95-106.

Glennon, KL., Rissler, LJ., & Church, SA. *In press*. Ecogeographic isolation: a reproductive barrier between species and between cytotypes in *Houstonia* (Rubiaceae). Evolutionary Ecology, *in press.*

Hendry, AP. 2009. Evolutionary Biology: Speciation. Nature 458: 162-164.

Hentrich, H., Kaiser, R., & Gottsberger, G. 2010. Floral biology and reproductive isolation by floral scent in three sympatric aroid species in French Guiana. Plant Biology 12: 587-596.

Husband, BC. 2004. The role of triploid hybrids in the evolutionary dynamics of mixed-ploidy populations. Biological Journal of the Linnean Society 82: 537-546.

Husband, BC. & Schemske, DW. 2000. Ecological mechanisms of reproductive isolation between diploid and tetraploid *Chamerion angustifolium*. Journal of Ecology 88: 689-701.

Husband, BC. & Sabara, HA. 2003. Reproductive isolation between autotetraploids and their diploid progenitors in fireweed, *Chamerion angustifolium* (Onagraceae). New Phytologist 161: 703-713.

Ireland, RR. 1957. Biosystematics of *Erythronium albidum* and *E. mesochoreum*. M.A. dissertation, University of Kansas, Lawrence.

Kaul, RB. 1989. The Status of *Erythronium albidum* and *E. mesochoreum* (Liliaceae) in Nebraska. Transactions of the Nebraska Academy of Sciences 17: 71-79.

Kay, KM. 2006. Reproductive isolation between two closely related hummingbird-pollinated Neotropical gingers. Evolution 60(3): 538-552.

Knerr, EG. 1891. *Erythronium mesochoreum*. Transactions of the Kansas Academy of Sciences 13: 20-21.

Lowry, DB., Modliszewski, JL., Wright, KM., Wu, CA. & Willis, JH. 2008. The strength and genetic basis of reproductive isolating barriers in flowering plants. Philosophical Transactions of the Royal Society B 363: 3009-3021.

Mallet, J. 1995. A species definition for the Modern Synthesis. Trends in Ecology and Evolution 10(7): 294-299.

Marks, GE. 1966. The origin and significance of intraspecific polyploidy: experimental evidence from *Solanum chacoense*. Evolution 20: 552-557.

Mathew, B. 1992. A taxonomic and horticultural review of *Erythronium* L. (Liliaceae). Botanical Journal of the Linnean Society 109: 453-471.

Mayr, E. 1942. *Systematics and the Origin of Species.* Columbia University Press, New York.

Mayrose, I., Zhan, SH., Rothlefs, CJ., Magnuson-Ford, K., Barker, MS., Rieseberg, LH., & Otto, SP. 2011. Recently formed polyploid plants diversify at lower rates. Science 333: 1257.

Michener, CD., & Rettenmeyer, CW. 1956. The ethology of *Andrena erythronii* with comparative data on other species (Hymenoptera, Andrenidae). The University of Kansas Science Bulletin 37: 645-684.

Mishler, BD. & Donoghue, MJ. 1982. Species concepts: a case for pluralism. Systematic Zoology 31(4): 491-503.

Muller, RN. 1979. Biomass accumulation and reproduction in *Erythronium albidum*. Bulletin of the Torrey Botanical Club 106 (4): 276-283.

Petit, C., Bretagnolle, F., & Felber, F. 1999. Evolutionary consequences of diploid-polyploid hybrid zones in wild species. Trends in Ecology and Evolution 14(8): 306-311.

Pires, JC. & Gaeta, RT. 2011. Structural and functional evolution of resynthesized polyploids. pp. 195-214 *in* Schmidt, R. & Bancroft, I. (eds.) *Genetics and Genomics of the Brassicaceae*. Springer, New York.

Pleasants, JM. & Wendel, JF. 1989. Genetic diversity in a clonal narrow endemic, *Erythronium propullans*, and its widespread progenitor, *Erythronium albidum*. American Journal of Botany 76(8): 1136-1151.

Ramsey, J. & Schemske, DW. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annual Review of Ecology and Systematics 29: 467-501.

Ramsey, J., Bradshaw, HD., & Schemske, DW. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). Evolution 57(7): 1520-1534.

Rieseberg, LH., Desrochers, AM., & Youn, SJ. 1995. Interspecific pollen competition as a reproductive barrier between sympatric species of *Helianthus* (Asteraceae). American Journal of Botany 82(4): 515-519.

Rieseberg, LH. & Willis, JH. 2007. Plant speciation. Science 317: 910-914.

Robertson, KR. 1966. The genus *Erythronium* (Liliaceae) in Kansas. Annals of the Missouri Botanical Garden 53(2): 197-204.

Schemske, DW., Willson, MF., Melampy, MN., Miller, LJ., Verner, L., Schemske, KM., & Best, LB. 1978. Flowering ecology of some spring woodland herbs. Ecology 59(2): 351-366.

Schmutz, J., Cannon, SB., Schlueter, J., Ma, J., Mitros, T., Nelson, W., Hyten, DL., Song, Q., Thelen, JJ., Cheng, J. *et al.* 2010. Genome sequence of the palaeopolyploid soybean. Nature 463: 178-183.

Schrader, MN. & LaBerge, WE. 1978. The nest biology of the bees *Andrena (Melandrena) regularis* Malloch and *Andrena (Melandrena) carlini* Cockerell (Hymenoptera: Andrenidae). Illinois Natural History Survey Biological Notes 108.

Schwenk, K., Brede, N., & Stret, B. 2008. Introduction. Extent, processes and evolutionary impact of interspecific hybridization in animals. Philosophical Transactions of the Royal Society B 363: 2805-2811.

Simpson, GG. 1961. *Principles of Animal Taxonomy*. Columbia University Press, New York.

Sobel, JM., Chen, GF., Watt, LR., & Schemske, DW. 2010. The biology of speciation. Evolution 64(2): 295-315.

Soltis, PS. & Soltis, DE. 2009. The role of hybridization in plant speciation. Annual Review of Plant Biology 60: 561-588.

Soltis, DE., Soltis, PS., & Tate, JA. 2003. Advances in the study of polyploidy since *Plant Speciation*. New Phytologist 161: 173-191.

Suda, J., Kron, P., Husband, BC., & Trávníček, P. 2007. Flow cytometry and ploidy: Applications in plant systematics, ecology and evolutionary biology. pp. 103-

131 *in* Dolezel, J., Greilhuber, J., & Suda, J. (eds.) *Flow Cytometry with Plant Cells: Analysis of Genes, Chromosomes, and Genomes*. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Trávníček, P., Dočkalová, Z., Rosenbaumová, R., Kubátová, B., Szelag, Z., & Chrtek, J. 2011. Bridging global and microregional scales: ploidy distribution in *Pilosella echioides* (Asteraceae) in central Europe. Annals of Botany 107(3): 443-454.

Van Valen, L. 1976. Ecological species, multispecies, and oaks. Taxon 25(2/3): 233-239.

Wang, X., Shi, X., Hao, B., Ge, S., & Luo, J. 2005. Duplication and DNA segmental loss in the rice genome: implications for diploidization. New Phytologist 165: 937-946.

Widmer, A., Lexer, C., & Cozzolino, S. 2009. Evolution of reproductive isolation in plants. Heredity 102: 31-38.

Wood, TE., Takebayashi, N., Barker, MS., Mayrose, I., Greenspoon, PB., & Rieseberg, LH. 2009. The frequency of polyploid speciation in vascular plants. Proceedings of the National Academy of Sciences 106(33): 13875-13879.

CHAPTER 2

ASSESSING THE FREQUENCY OF HYBRIDIZATION BETWEEN *ERYTHRONIUM ALBIDUM* AND ITS CONGENER *E. MESOCHOREUM* (LILIACEAE)

INTRODUCTION

Hybrid zones serve as excellent natural laboratories for studying fundamental processes in evolutionary biology—including reproductive isolation, speciation, the evolutionary consequences of ecological interactions, and patterns of gene flow between closely related taxa (Hewitt 1988, Cruzan & Arnold 1994, Peñaloza-Ramírez *et al.* 2010). Understanding the frequency of hybridization and the mechanisms limiting or preventing it among closely related taxa is integral to the study of speciation (Barton & Hewitt 1989, Petit *et al.* 1999).

Speciation occurs when diverging populations become reproductively isolated from one another (biological species concept; Dobzhansky 1935, Mayr 1942). Reproductive barriers limit or preclude interspecific gene flow, which is initiated by the formation of fertile hybrids (Coyne & Orr 2004). Reproductive isolation generally occurs through the action of multiple isolating barriers (Coyne & Orr 2004, Rieseberg & Willis 2007). Among plants, examples of such barriers include prezygotic mechanisms such as habitat-based spatial isolation (Nagy & Rice 1997, Ramsey *et al.* 2003) and flowering asynchrony (Vasek & Sauer 1971, Pascarella 2007), as well as postzygotic mechanisms such as hybrid inviability and

sterility (Rieseberg 1997, Lopez *et al.* 2000). Understanding the distribution and relative frequencies of hybrids and their parental species at contact zones can shed valuable insight into the nature and degree of reproductive isolation between closely related plant taxa (Harrison & Rand 1989).

During the process of speciation, diverging populations become progressively more distinct, and additional pre- and postzygotic barriers to gene flow develop. As a result, the extent of natural hybridization diminishes (Grant 1971). In contrast, the presence of fertile hybrids indicates that species boundaries between two taxa are incomplete. If fertile hybrids are common, they can erode reproductive isolation by acting as a bridge through which genes can be transferred from one species to another, thereby reducing their morphological and genetic distinctiveness (Grant 1971). However, it is important to note that hybrids can also be formed between members of well-established biological species, and may continue to occur millions of years after the initial divergence between species (Coyne & Orr 2004, Mallet 2005).

Understanding patterns of hybridization is especially important with regard to triploid hybrids formed between diploid-tetraploid plant species pairs and cytotypes. Triploid hybrids play an important role in the evolutionary dynamics among diploids and tetraploids, though whether they facilitate polyploid speciation or limit it is a subject of debate (Husband 2004). In many plant taxa, triploids are inviable or sterile (Dweikat & Lyrene 1988, Lumaret & Barrientos 1990, Petit *et al.* 1999). This phenomenon, termed triploid block, often arises because of seed developmental abnormalities and the formation of aneuploid gametes (Ramsey &

Schemske 1998). Among established diploid-tetraploid species pairs, low-fitness triploids limit the extent of interspecific gene flow, thus maintaining reproductive isolation. The role that triploids play in the initial phases of polyploid speciation is less clear.

The formation of tetraploid individuals and the establishment of tetraploid populations are the first stages in polyploid speciation. Tetraploids arising within diploid populations are initially outnumbered by their diploid progenitors and suffer reduced fitness due to a process called minority cytotype exclusion (Levin 1975, Husband 2004). Under these circumstances, establishing tetraploids primarily mate with diploids, as diploids are more numerous. The resultant sterile or inviable triploids constitute a reproductive "dead end". The formation of these low-fitness triploids inhibits tetraploid establishment, thus slowing or halting the speciation process.

However, recent surveys indicate that triploid hybrids across many plant families can retain at least partial viability and fertility (Ramsey & Schemske 1998, Husband 2004). In addition, recent mathematical models have indicated that even low-fitness triploids may play a key role in promoting polyploid speciation by producing unreduced gametes that facilitate the establishment of tetraploids (Felber & Bever 1997, Husband 2004).

Because of the uncertainty regarding triploids' roles in evolutionary processes among diploids and polyploids, identifying patterns and frequencies of hybrid occurrence among mixed-ploidy species pairs is key to improving our understanding of reproductive isolation and speciation in mixed-ploidy plant

systems. Nevertheless, we still have insufficient information regarding patterns and frequencies of triploid hybrids in areas where mixed-ploidy species pairs come into contact with one another (Ramsey & Schemske 1998, Husband 2004).

Erythronium albidum Nutt. and *Erythronium mesochoreum* Knerr (Liliaceae) are well-suited to studies of hybridization and reproductive isolation for several reasons. *E. albidum* and *E. mesochoreum* are sympatric: *E. mesochoreum*'s geographic range is restricted to the Midwestern United States, whereas *E. albidum*'s range extends from central U.S. states to the east coast (Allen & Robertson 2002, Figure 1.3—Chapter 1). In addition, although they tend to occupy different types of habitats, populations of *E. albidum* and *E. mesochoreum* can come into close contact where their habitats abut (Churchill 1986, Kaul 1989). Both species share a base chromosome number of x = 11, but differ in ploidy. This disparity in genome size allows *E. albidum*, *E. mesochoreum*, and hybrid individuals to be readily distinguished using flow cytometry. Furthermore, both karyotype analyses and genetic sequence data strongly suggest that *E. albidum* and *E. mesochoreum* are sister species (Allen *et al.* 2003). Finally, one account of hybridization between these species has previously been recorded (R.B. Kaul, unpublished data), indicating that reproductive boundaries between *E. albidum* and *E. mesochoreum* may be incomplete and of relatively recent origin.

The goal of this study was to quantify the frequency of hybridization between *E. albidum* and *E. mesochoreum* at four contact zones. I used targeted, non-random sampling of plants with intermediate morphological characteristics to assess whether hybrids were present at each site. To quantify the abundance of hybrids

relative to parental species, I also systematically sampled plants across each contact zone. The ploidy of each plant was identified using flow cytometry. My results indicate that hybrid individuals are uncommon and that reproductive isolation between *E. albidum* and *E. mesochoreum* is strong.

MATERIALS AND METHODS

Study Species

E. albidum Nutt. and *E. mesochoreum* Knerr (Liliaceae) are perennial forbs belonging to the eastern North American clade of the genus *Erythronium* (Allen *et al.* 2003). *E. albidum* is widely distributed, ranging from the Midwest to the eastern U.S. and from the southern U.S. north into Ontario, Canada (Allen & Robertson 2002). *E. mesochoreum*'s range is much narrower and is limited to central U.S. states (Allen & Robertson 2002). Although *E. mesochoreum* is diploid (2n = 2x = 22), and *E. albidum* is tetraploid (2n = 4x = 44), both species have a base chromosome number of x = 11 (Robertson 1966). Because of this difference in ploidy, *E. albidum—E. mesochoreum* hybrids are likely triploid. Additionally, karyotype evidence and sequence data from the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA genes strongly suggest that *E. albidum* and *E. mesochoreum* are sister species (Allen *et al*. 2003). Although the relationship among *E. albidum*, *E. mesochoreum*, and a closely related species, *E. umbilicatum*, could not be resolved using ITS sequence data, *E. umbilicatum* and the rest of the *Erythronium* genus share a base chromosome number of x = 12 (with the exception of *E. propullans,* which is endemic to two counties in Minnesota and is likely sustained almost entirely by

vegetative reproduction; Banks 1980). The most parsimonious explanation for these patterns of ploidy is that the base chromosome number of x = 11 was derived only once, and that *E. albidum* and *E. mesochoreum* are sister species.

Both species are monoecious spring ephemerals (Churchill 1986). Although *E. albidum* is primarily found in wooded areas and *E. mesochoreum* predominantly inhabits tallgrass prairie, populations of these species can abut at prairie-forest borders and can intergrade more widely in areas such as oak savannahs (Robertson 1966, Kaul 1989, McClain 1999).

Several morphological characteristics can be used to distinguish *E. albidum* from *E. mesochoreum*. Notably, *E. mesochoreum*'s leaves are usually conduplicate and unmottled, whereas *E. albidum* typically has darkly mottled leaves that are only slightly folded (Figure 1.5—Chapter 1; Churchill 1986). However, these characteristics can vary among populations (Rickett 1937, K. Roccaforte, personal observation), which can make the species difficult to distinguish from one another in areas where they co-occur. This, in turn, makes the identification of hybrids based on intermediate morphology challenging.

I conducted a survey of 635 *E. albidum* and *E. mesochoreum* accessions at 4 Midwestern herbaria (Table 2.1) and found one accession labeled as a hybrid (NEB 306411). The accession consists of three flowering plants and one single-leaved individual collected on April 5, 1995. Karyotypes of the four plants comprising the herbarium accession, performed at the time of collection, revealed an intermediate number of chromosomes (R.B. Kaul, unpublished data). However, an exact chromosome count was not feasible due to the size and large number of

chromosomes (R.B. Kaul, personal communication). Subsequent flow cytometry analyses conducted in 1995 confirmed that these plants had a genome size larger than *E. mesochoreum*, yet smaller than *E. albidum*, which was taken as evidence that the four plants were hybrids (R.B. Kaul, unpublished data).

Study Sites

I conducted sampling at four Midwestern contact zones— Tallgrass Prairie National Preserve in Chase Co., KS, Yellow Smoke Park in Crawford Co., IA, MacLennan Park in Shawnee Co., KS., and Red Cedar Recreation Area in Saunders Co., NE (Figure 1.6—Chapter 1; Table 2.2).

Tallgrass Prairie National Preserve (TPNP) is a 4500 ha preserve located in the Flint Hills region of east-central Kansas (Chase Co.). TPNP is managed cooperatively by the Nature Conservancy and the National Park Service. The sample site was located on a steep, north-facing slope with exposed limestone outcroppings. Common trees found in this region include bur oak, American elm, black willow, and Osage orange. Silt loam soils predominate at the contact zone (Ivan silt loam; Soil Survey Staff NRCS-USDA 2012).

Yellow Smoke Park is a 145 ha park located in Crawford Co., IA that is operated by the Crawford County Conservation Board. The contact zone at Yellow Smoke is located on a west-facing slope in a woodland comprised of bur oak, ironwood, bitternut hickory, and green ash. Understory plants include Solomon's seal, columbine, snow trillium, Dutchman's breeches, and Virginia waterleaf. Silt loam and clay loam soils predominate at the contact zone (Knox silt loam, Liston-Burchard complex; Soil Survey Staff NRCS-USDA 2012). Historically, this site

consisted of a virgin prairie (containing *E. mesochoreum*) directly abutting undisturbed woodland (containing *E. albidum*). The prairie was grazed heavily and eventually planted with brome. After the Crawford County Conservation Board acquired the land, grazing was halted and the woodland encroached onto the remainder of the prairie (G. Pollock, personal communication).

MacLennan Park is a 90 ha wildlife refuge located along the banks of the Kansas River in Topeka, KS (Shawnee Co.). The sample site was located along a north-facing slope in a forest dominated by bur oak, chinkapin oak, shagbark hickory, and bitternut hickory. Understory plants at this site include Virginia creeper, garlic mustard, wild blue phlox, dutchman's breeches and bedstraw. The predominant soil type at the contact zone is silty clay loam (Vinland-Rock outcrop complex; Soil Survey Staff NRCS-USDA 2012).

Red Cedar Recreation area is a 70 ha public use facility in Saunders Co., NE that is operated by the Lower Platte South Natural Resources District. The sample site was located on an east-facing slope in a relatively undisturbed woodland of bur oak, American and red elms, green ash, and black walnut. The understory consists of several species of woodland sedge, as well as dutchman's breeches and Jack in the pulpit (R.B. Kaul, personal communication). Silt loam soils predominate at the contact zone (Ida-Steinauer complex; Soil Survey Staff NRCS-USDA 2012). A large colony of *Erythronium albidum* occurs along the hill slopes and in the ravines of this site. An upland tallgrass prairie historically bordered these ridges and was likely dominated by big bluestem, little bluestem, and porcupine needlegrass. Although a larger population of *Erythronium mesochoreum* likely once existed in this prairie,

land use changes have restricted its current distribution to the top of the forested ridges of Red Cedar.

Field Sampling at Contact Zones

In 2010 and 2011, I used flow cytometry to assess whether 128 *Erythronium* individuals with intermediate morphological traits (primarily leaf folding and mottling) were hybrids. Sampling was conducted at all four contact zones (Table 2.2).

Next, to assess the frequency of hybrid plants in areas where *E. mesochoreum* and *E. albidum* co-occur, in spring 2011 I systematically collected 224 *Erythronium* leaves at three of these contact zones (Table 2.2). At each site I established several parallel transects that spanned the zone of species contact. Depending on the size of the contact zone, 5-9 parallel transects were established, and the transects ranged from 10-40 meters in length. I collected the nearest *Erythronium* leaf every two meters along each transect. At each site, if there were no *Erythronium* leaves present within a one meter radius of the designated collection point, that point was omitted, and I moved to the next collection point. Prior to harvesting, each study plant was photographed, and its morphological characteristics and phenological status were noted. Of the 224 leaves collected across these sites, ten leaves were unable to be analyzed using flow cytometry, likely because the quality of the tissue had degraded. These plants were categorized as either *E. mesochoreum* or *E. albidum* based on morphological characteristics that were recorded at the time of collection. Immediately after collection, the leaves were placed in plastic zip-top bags containing silica gel to ensure rapid drying. I stored the leaves under ambient

conditions in the laboratory, and the silica gel was changed periodically to ensure that the leaves remained dry.

Identification of Hybrids

I used flow cytometry to identify hybrids between *E. albidum* and *E. mesochoreum*. Flow cytometry allows rapid and accurate measurement of nuclear DNA content, whether in absolute units (*i.e.* picograms, base pairs) or relative to a reference species (Greilhuber *et al*. 2007). Because *E. albidum* and *E. mesochoreum* are closely related, share the same base chromosome number, and differ in ploidy, they are well-suited to identification based on genome size differences using flow cytometry. I anticipated that *E. albidum*'s nuclear DNA content would be approximately double that of *E. mesochoreum*, and that DNA content for hybrids would be intermediate to that of the parental species.

I processed approximately 4 cm^2 of dried *Erythronium* leaf tissue per sample for flow cytometry, along with approximately 2 cm^2 *Allium cepa* cv. 'Ailsa Craig' as an internal reference standard (Bennett *et al.* 2000). The leaf tissues of the sample and standard were co-chopped in 1-2 mL cold Galbraith's buffer (Galbraith *et al.* 1983) with 0.2-0.5% vol/vol Triton X-100 detergent. The suspension was filtered through a 50 μm nylon filter, and the filtrate was centrifuged for 5 minutes at 2400*x*g. I then discarded the supernatant and resuspended the pellet in 500 μL Galbraith's buffer. Ten minutes prior to analysis, I added 75 μL RNase solution (0.5 $mg \bullet mL^{-1}$) and 50 μL propidium iodide solution (1.0 $mg \bullet mL^{-1}$) to the suspension. The prepared samples were kept at 4°C prior to cytometry. The samples were

analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and I recorded $\geq$ 5000 events per run. Relative fluorescence (RF) was calculated by dividing the mean fluorescence intensity of the sample peak by the mean fluorescence intensity of the standard (*Allium cepa*) peak. Low leaf tissue quality for *E. albidum* required me to record fluorescence intensity on a log scale. Therefore, no coefficients of variation (CV) values were calculated for the peaks (Doležel *et al.* 2007).

To establish a reference range of RF values for *E. albidum*, *E. mesochoreum*, and hybrid samples, I collected 56 known *E. mesochoreum* plants and 54 known *E. albidum* plants in 2010 and 2011 from seven sites in Nebraska and Iowa where the study species do not co-occur (Table 2.3). I processed the leaves as outlined above and calculated the RF of each sample, to establish a range of known RF values for *E. albidum* and *E. mesochoreum.* Each leaf tissue sample subsequently collected from the contact zones was classified as *E. albidum* or *E. mesochoreum* if its RF value fell within the range of reference RF values for that species. I classified a sample as "hybrid" if its RF value fell between the mean of the lowest known *E. albidum* and *E. mesochoreum* RF values, and the mean of the highest known *E. albidum* and *E. mesochoreum* RF values (modified from Husband & Schemske 1998). The number of *E. albidum*, *E. mesochoreum*, and hybrid individuals was then tallied for each site.

RESULTS

Relative Fluorescence (RF) Ranges of E. albidum and E. mesochoreum

The 54 known *E. albidum* leaf tissue samples had a mean (± 1 standard error) RF value of 4.53 ± 0.01 (range: 4.22-4.70), whereas the 56 known *E. mesochoreum* leaf tissue samples had a mean (± 1 standard error) RF value of 3.02 ± 0.02 (range: 2.62-3.25) (Figure 2.1). I therefore established the expected range of RF values for hybrid individuals as 3.42-3.98 (Figure 2.1). I was able to classify 87% of the leaves collected from contact zones as *E. albidum*, *E. mesochoreum*, or hybrid based on these RF value ranges, indicating that flow cytometry was an appropriate method of identifying *Erythronium* leaves. Notably, leaves with RF values that fell outside of these measured or predicted ranges had RF values that fell either slightly above the highest *E. albidum* RF value or slightly below the lowest *E. mesochoreum* RF value (with one exception; see below). Therefore, ambiguities regarding hybrid identification based on intermediate RF values that did not fall within the predicted range for hybrids were largely avoided. Of the leaves collected from contact zones, one leaf's RF value fell below the range of known *E. mesochoreum* RF values (RF = 2.59). This leaf was classified as *E. mesochoreum*. Forty-four leaves collected across the contact zones had RF values slightly higher than the known *E. albidum* RF values. The mean RF (± 1 standard error) of these leaves was 4.79 ± 0.01. These leaves were classified as *E. albidum*.

Extent of Hybridization between E. albidum and E. mesochoreum

Of the 128 morphologically intermediate individuals sampled, I confirmed that eight were hybrids because their DNA content fell within the expected RF range for hybrids. Mean RF (± 1 standard error) for these hybrid individuals was 3.91 ± 0.01 (range: 3.85-3.97). All of the hybrid individuals had light green or white leaf

mottling, and they were found exclusively at Red Cedar Recreation Area, approximately 20 meters east of the area where I carried out the systematic sampling (Figure 2.2).

Despite the presence of hybrids at Red Cedar Recreation Area, the systematic sampling of plants across contact zones at three sites revealed no conclusive hybrids. At Yellow Smoke Park, I systematically collected 65 leaves. Both *E. mesochoreum* and *E. albidum* were present at the contact zone, but none of the leaves were identified as hybrids (Figure 2.3A). Overall, 17 leaves were identified as *E. albidum*, including one individual that was not successfully identified using flow cytometry and was assessed based on morphology. *E. albidum* individuals were found primarily at the southern end of the site. The remaining 48 leaves were identified as *E. mesochoreum*, and these individuals were more common at the northern end of the site.

At MacLennan Park, 45 leaves were systematically collected across the contact zone. Of these, four individuals (three *E. albidum* leaves and one *E. mesochoreum* leaf) were not successfully identified using flow cytometry and were thus classified on the basis of leaf morphology. Although I confirmed the presence of both *E. albidum* and *E. mesochoreum* individuals at this site, none of the leaves I collected came from hybrid individuals (Figure 2.3B). A total of 15 leaves were classified as *E. albidum*, and these plants were predominantly located at the northwest corner of the study site. The remaining 30 leaves were classified as *E. mesochoreum*.

At Red Cedar Recreation Area, I systematically collected 114 leaves. Of these leaves, 52 were confirmed to be *E. albidum*, and 56 were confirmed to be *E. mesochoreum* using flow cytometry (Figure 2.3C). Five leaves were identified using morphological characteristics—three were classified as *E. albidum*, and two were classified as *E. mesochoreum*. Overall, *E. mesochoreum* individuals were more common on the western half of this site, whereas *E. albidum* individuals were predominantly found on the eastern half of the site. One individual from this site had a RF value of 4.04, which is slightly above the highest expected RF value for hybrid individuals, yet falls below the lowest recorded *E. albidum* RF value. This single-leaved plant displayed intermediate leaf morphology. Its leaf resembled *E. albidum* in that it was flat, but it lacked the mottling that is typical of *E. albidum* leaves. Therefore, it is likely that this leaf was of hybrid origin.

DISCUSSION

Even though *E. albidum* and *E. mesochoreum* are putative sister species with overlapping geographic ranges, I found no evidence of widespread hybridization between these taxa, based on extensive sampling at four contact zones. Hybrids were only identified at one of the four contact zones (Red Cedar Recreation Area). However, it must be noted that I sampled more extensively at this site, due to its proximity to the University of Nebraska. It may be the case that hybrids are present, though not abundant, at the other contact zones. Nevertheless, my results suggest that reproductive isolation is strong and that gene flow between *E. albidum* and *E. mesochoreum* is likely fairly uncommon in nature.

Potential Reproductive Barriers

There are several mechanisms by which these species may be reproductively isolated from one another, and closely related plant species are generally isolated from each other via multiple reproductive barriers and these barriers' interactions (Widmer *et al.* 2009). Strong reproductive isolation between diploid-tetraploid plant species and cytotypes is generally believed to stem from triploid block. Triploid block has been viewed as a widespread phenomenon, but this view is likely an overgeneralization (Husband 2004). Indeed, triploid hybrids across many plant taxa have been shown to retain viability and fertility, though they often exhibit reduced fitness when compared to parental species (Ramsey & Schemske 1998).

Five of the eight hybrid plants that I located at Red Cedar Recreation Area were in flower, indicating that *E. albidum*-*E. mesochoreum* hybrids are capable of retaining viability and surviving to reproductive maturity. It is also likely that hybrids are capable of asexual reproduction, as vegetative propagation occurs for both parental species and is especially commonplace for *E. albidum* (Muller 1979). However, it remains unclear how overall hybrid fitness, including germination, growth, and reproduction, compares to the fitness of the parental species. I noted the presence of pollen on the anthers of hybrid plants, and insects (likely females of the solitary bee species *Andrena carlini*, the primary pollinators of *E. albidum*; Banks 1980) were observed visiting hybrid flowers. I do not know, however, whether hybrid pollen is fertile. Hybrids that produce fertile pollen have the potential to backcross with members of the parental species and could erode reproductive

isolation between *E. albidum* and *E. mesochoreum*, although my study indicates that opportunities for this would be few.

In addition to triploid block, other reproductive barriers such as habitat-based spatial isolation, flowering asynchrony and numerous forms of pollinator-based isolation can impart reproductive isolation between diploid and polyploid cytotypes and species pairs (Segraves & Thompson 1999, Petit *et al.* 1999, Husband & Sabara 2003). The role that these barriers play in the origin and maintenance of species boundaries between diploid-polyploid plant taxa has not been thoroughly studied (Husband & Sabara 2003). My investigation of the extent of hybridization between *E. albidum* and *E. mesochoreum* is the first stage of a larger study I am conducting to assess the contributions of multiple isolating mechanisms—including flowering asynchrony, pollinator-based isolation, and post-pollination physiological barriers—to the maintenance of species boundaries between these putative sister species.

Spatial Distributions of E. albidum and E. mesochoreum at Contact Zones

The systematic sampling across contact zones demonstrated that both species aggregated with conspecifics. The clumped spatial distribution of both *E. albidum* and *E. mesochoreum* at each of these sites likely reflects the interplay of several processes, including vegetative reproduction, seed dispersal, adaptations to microhabitats within each contact zone, and site history.

Both *E. albidum* and *E. mesochoreum* reproduce asexually, via stolons and bulb offshoots, respectively (Churchill 1986, Kaul 1989). Widespread vegetative reproduction allows *E. albidum* to form dense stands consisting primarily of sterile,

one-leaved individuals. Muller (1979) found that over 99% of the *E. albidum* plants he surveyed in a population in northeastern Illinois were non-flowering, and that 45% of *E. albidum* plants he surveyed reproduced vegetatively during the study year. Further, new individuals (corms) resulting from vegetative reproduction had an overwinter survival rate of 99%. In contrast, only 0.4% of *E. albidum* plants he surveyed were flowering, and less than 0.1% of plants were seedlings. Taking into account biomass allocation and rates of successful establishment, Muller (1979) estimated that sexual reproduction was far more costly (~ 14 fold) than vegetative reproduction, suggesting that vegetative reproduction may often be a more successful reproductive strategy for *E. albidum*. The reproductive biology of *E. mesochoreum* is less well understood. It has been documented that non-flowering individuals are less common in *E. mesochoreum* populations, when compared to populations of *E. albidum* (Knerr 1891, Kaul 1989). Furthermore, seed production is generally higher for *E. mesochoreum* than for *E. albidum* (Churchill 1986). Taken together, these observations suggest that *E. mesochoreum* may utilize vegetative reproduction less often than *E. albidum.* Overall, *E. mesochoreum* populations tend to be less dense than *E. albidum* populations (Kaul 1989), and I confirmed this observation at my study sites. Nevertheless, my systematic sampling revealed that both species generally aggregated with conspecifics.

Patterns and ranges of seed dispersal for *E. albidum* and *E. mesochoreum* remain unclear, but some broad inferences can be made based on seed morphology. Both species produce seeds bearing elaiosomes (Churchill 1986), indicating that ants are at least partially responsible for seed dispersal. In a global survey of

myrmecochores, Gómez & Espadaler (1998) found that the average dispersal distance for ant-dispersed seeds was 0.96 meters, which is much shorter than dispersal distances generated by other animals. It is also possible that large herbivores such as white-tailed deer occasionally effect long-distance dispersal of *Erythronium* seeds (Myers *et al.* 2004). Nevertheless, the majority of *E. albidum* and *E. mesochoreum* seeds are likely not dispersed very far, which could contribute to clumped recruitment patterns at the study sites.

The spatial distributions of *E. albidum* and *E. mesochoreum* at the contact zones likely also reflect adaptations to particular habitats within each site. *E. albidum* typically inhabits wooded areas and mesic ravines whereas *E. mesochoreum* is generally restricted to prairies and upland, lightly wooded slopes (Ireland 1957, Robertson 1966).

These patterns, however, may be blurred by the rapid land use changes that have occurred in central U.S. states over the past 150 years. All of the contact zones I surveyed were wooded, and it is likely that fire suppression and other land-use changes have fostered the growth of woody species at each of these sites. However, when one takes into account these land-use changes, microhabitat based patterns of spatial segregation of *E. albidum* and *E. mesochoreum* become more apparent.

At Red Cedar Recreation Area, the majority of *E. mesochoreum* plants I sampled were located near the top of a slope. Though the slope is now wooded and is bordered by a hayfield, this area was historically adjacent to an upland tallgrass prairie (R.B. Kaul, personal communication). The majority of *E. albidum* individuals that I identified were located near the bottom of the slope, in a mesic ravine that is

more heavily wooded. At Yellow Smoke Park, *E. mesochoreum* was predominantly found in the northern region of the contact zone. Though the entire contact zone at Yellow Smoke Park is currently wooded, the northern area of the contact zone lacked tree cover until approximately 1960. *E. albidum* individuals at this site were found primarily at the southern edge of the contact zone, in an area that has historically been wooded (G. Pollock, personal communication). The influences of both site history and microhabitat adaptation were less clear at MacLennan Park and Tallgrass Prairie National Preserve. At Tallgrass Prairie National Preserve, the populations of *E. albidum* and *E. mesochoreum* were located approximately 75 meters apart. Because of the relatively large distance between the populations, I did not conduct systematic sampling at this site. At MacLennan Park, the systematic sampling was conducted over a fairly small (8 m x 10 m) area, and the land-use history of this small site is unclear. Although *E. mesochoreum* and *E. albidum* individuals were often located within 1-2 meters of each other at all contact zones, the clear spatial segregation of these species at each of my study sites has the potential to limit interspecific pollen transfer. This may represent a reproductive barrier, particularly if pollinators only travel short distances between flowers when foraging.

Conclusions

The limited occurrence of hybrids between *E. albidum* and *E. mesochoreum* at my study sites indicates that reproductive isolation between these taxa is strong. To more fully understand the nature of species boundaries between these species, the contributions of multiple reproductive barriers, such as flowering asynchrony,

pollinator assemblage overlap, and hybrid inviability and sterility need to be evaluated (Chapter 3). This knowledge will further our understanding of the nature of species boundaries between closely related plant taxa, especially with regard to diploid-polyploid plant species pairs.

TABLES

Table 2.1: Locations of herbaria surveyed to investigate the occurrence of hybrids between *E. albidum* and *E. mesochoreum.*

Herbarium	University	Location
Charles E. Bessey Herbarium (NEB)	University of Nebraska-Lincoln	Lincoln, NE
Ronald L. McGregor Herbarium (KANU)	University of Kansas	Lawrence, KS
Kansas State University Herbarium (KSC)	Kansas State University	Manhattan, KS
Ada Hayden Herbarium (ISC; IA)	Iowa State University	Ames, IA

Table 2.2: Collection locations and sample sizes for leaves collected via systematic sampling (SS) of *Erythronium* plants and for the collection of morphologically intermediate (MI) plants in 2010 and 2011.

Site	Location	Latitude	Longitude	Leaves Collected (MI)	Leaves Collected (SS)
MacLennan Park	Shawnee Co., KS	39.067° N	95.733° W	18	45
Red Cedar Recreation Area	Saunders Co., NE	41.169° N	96.880° W	77	114
Tallgrass Prairie National Preserve	Chase Co., KS	38.492° N	96.589° W	14	0
Yellow Smoke Park	Crawford Co., IA	42.024° N	95.323° W	19	65

Table 2.3: Collection locations of *Erythronium albidum* (EA) and *Erythronium mesochoreum* (EM) plants that were used to establish relative fluorescence values for both study species and hybrids. The numbers in bold represent the total sample size, and the numbers in parentheses represent leaves collected in 2010 and 2011, respectively.

Site	Location	Latitude	Longitude	Species	Leaves Collected	
Bur Oak Wildlife Management Area	Seward Co., NE	40.896° N	97.000° W	EA	**46**	(17,29)
Pioneers Park	Lancaster Co., NE	40.772° N	96.772° W	EA	**7**	(7,0)
woodland near Omaha, NE	Douglas Co., NE	41.362° N	95.968° W	EA	**1**	(1,0)
Bauermeister Prairie	Douglas Co., NE	41.215° N	96.166° W	EM	**3**	(3,0)
Buck Grove Cemetery	Crawford Co., IA	41.907° N	95.384° W	EM	**2**	(2,0)
Madigan Prairie	Saunders Co., NE	41.169° N	96.881° W	EM	**41**	(11,30)
Te Amo Prairie	Douglas Co., NE	41.192° N	96.208° W	EM	**10**	(10,0)

FIGURES

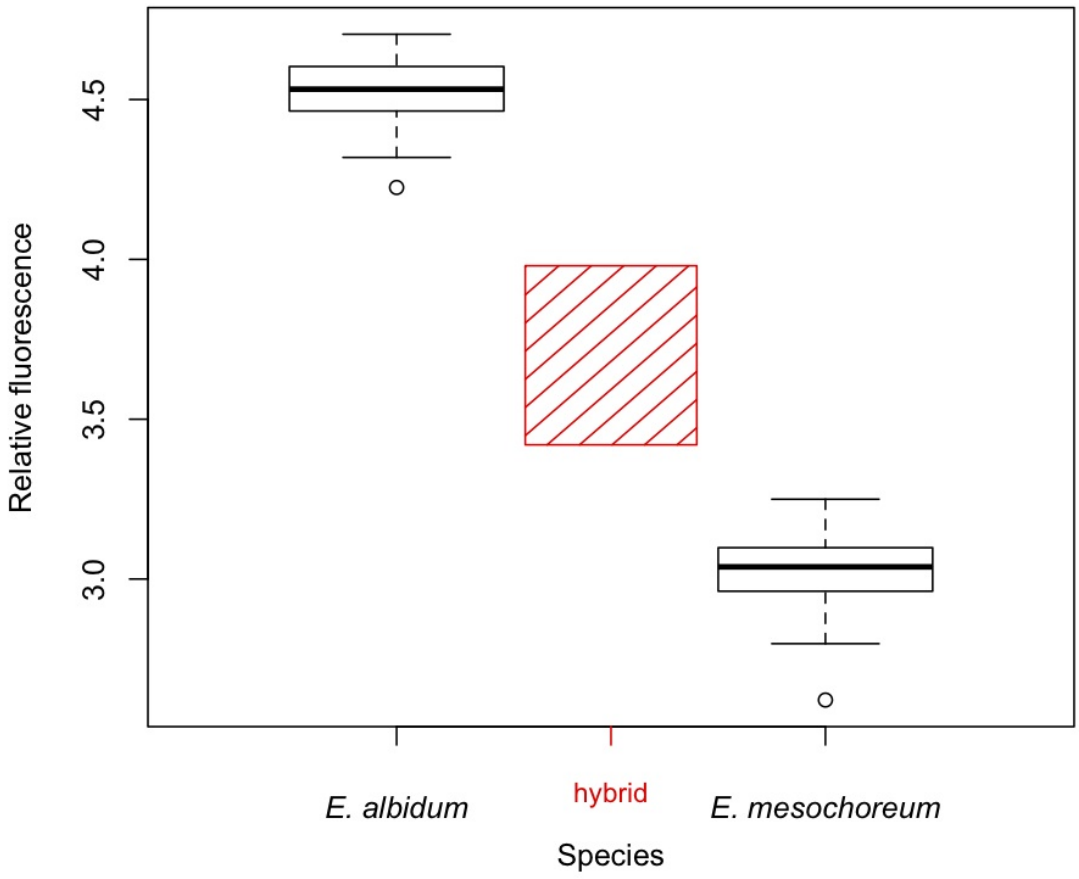

Figure 2.1: Relative fluorescence values (unitless, fluorescence relative to *Allium cepa* cv. 'Ailsa Craig') from flow cytometry for known *E. albidum* (n = 54) and *E. mesochoreum* (n = 56) individuals collected across 7 sites in the Midwestern U.S. in 2010 and 2011. The red, hashed area indicates the predicted range of relative fluorescence values for hybrids.

Figure 2.2: Four *E. albidum-E. mesochoreum* hybrid individuals, confirmed by genome size measurement using flow cytometry, at Red Cedar Recreation Area in Saunders Co., NE. Photo: K. Roccaforte.

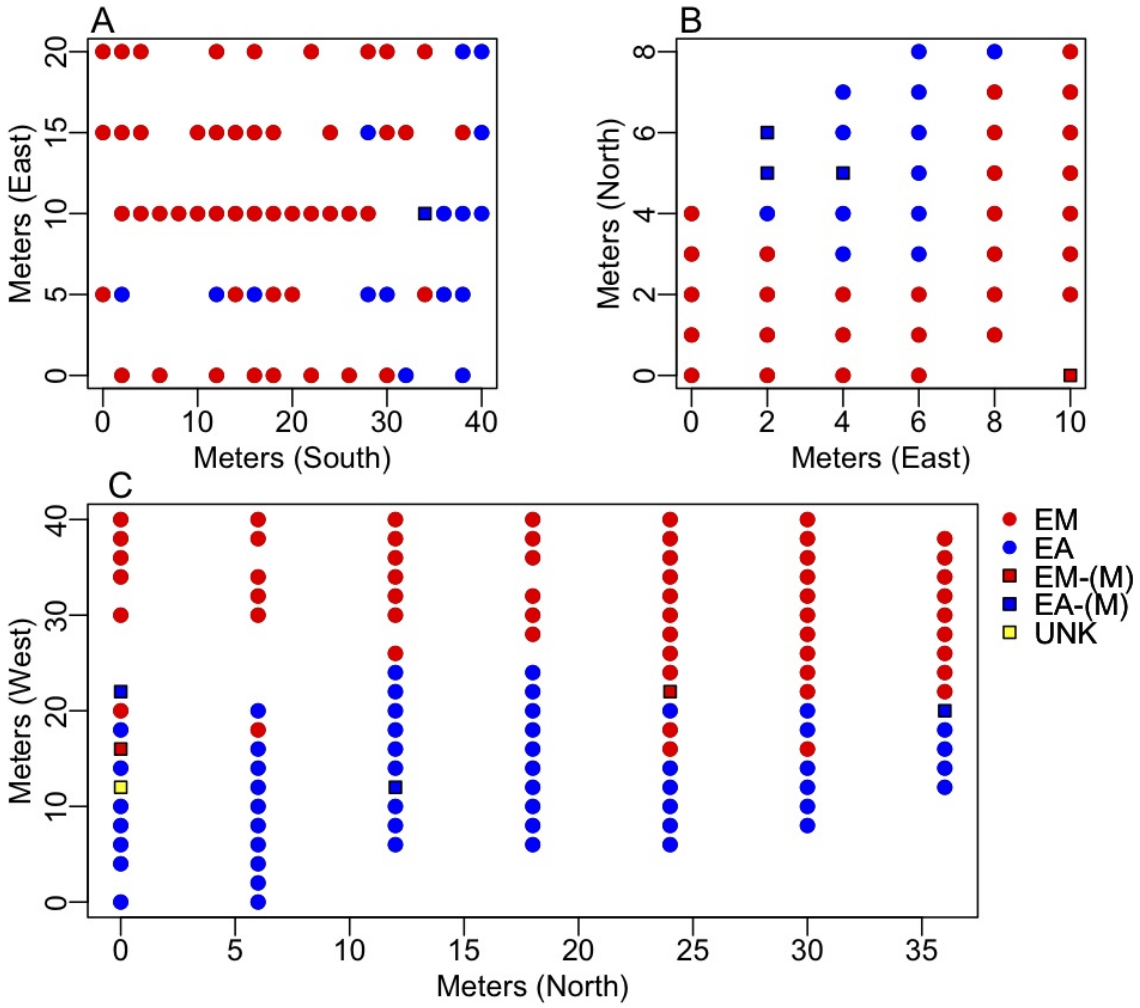

Figure 2.3: *E. albidum* and *E. mesochoreum* individuals identified using flow cytometry (EA and EM) or leaf morphology (EA-M, EM-M). Leaves were collected along parallel transects that were laid across contact zones at, **A.** Yellow Smoke Park (Crawford Co., IA); **B.** MacLennan Park (Shawnee Co., KS); **C.** Red Cedar Recreation Area (Saunders Co., NE). One leaf at this site (UNK) had a relative fluorescence value higher than the predicted range for hybrids, but lower than the measured range for *E. albidum*.

REFERENCES

Allen, GA. & Robertson, KR. 2002. Erythronium. pp. 153-164 *in* Flora of North America Editorial Committee (eds.) *Flora of North America, Vol. 26.* Oxford University Press, New York.

Allen, GA., Soltis, DE., & Soltis, PS. 2003. Phylogeny and biogeography of *Erythronium* (Liliaceae) inferred from chloroplast *matK* and nuclear rDNA ITS sequences. Systematic Botany 28(3): 512-523.

Banks, JA. 1980. The reproductive biology of *Erythronium propullans* Gray and sympatric populations of *E. albidum* Nutt. (Liliaceae). Bulletin of the Torrey Botanical Club 107: 181-188.

Barton, NH. & Hewitt, GM. 1989. Adaptation, speciation and hybrid zones. Nature 341: 497-503.

Bennett, MD., Johnston, S., Hodnett, GL., & Price, HJ. 2000. *Allium cepa* L. cultivars from four continents compared by flow cytometry show nuclear DNA constancy. Annals of Botany 85: 351-357.

Churchill, SP. 1986. Liliaceae Juss., the Lily Family. pp. 1241-1258 *in* Great Plains Flora Association (eds.) *Flora of the Great Plains*. University Press of Kansas, Lawrence, KS.

Coyne, JA. & Orr, HA. 2004. *Speciation*. Sinauer Associates, Sunderland, MA.

Cruzan, MB. & Arnold, ML. 1994. Assortative mating and natural selection in an *Iris* hybrid zone. Evolution 48(6): 1946-1958.

Dobzhansky, T. 1935. A critique of the species concept in biology. Philosophy of Science 2: 344-355.

Doležel, J., Greilhuber, J., & Suda, J. 2007. Estimation of nuclear DNA content in plants using flow cytometry. Nature Protocols 2(9): 2233-2244.

Dweikat, IM. & Lyrene PM. 1988. Production and viability of unreduced gametes in triploid interspecific blueberry hybrids. Theoretical and Applied Genetics 76: 555-559.

Felber, F. & Bever, JD. 1997. Effect of triploid fitness on the coexistence of diploids and tetraploids. Biological Journal of the Linnean Society 60: 95-106.

Galbraith, DW., Harkins, KR., Maddox, JM., Ayres, NM., Sharma, DP., & Firoozabady, E. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220 (4601): 1049-1051.

Gómez, C. & Espadaler, X. 1998. Myrmecochorous dispersal distances: a world survey. Journal of Biogeography 25: 573-580.

Grant, V. 1971. *Plant Speciation.* Columbia University Press, New York.

Greilhuber, J., Temsch, EM., & Loureiro, JCM. 2007. Nuclear DNA Content Measurement. pp. 67-101 *in* Dolezel, J., Greilhuber, J., & Suda, J. (eds.) *Flow Cytometry with Plant Cells: Analysis of Genes, Chromosomes, and Genomes*. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Harrison, RG. & Rand, DM. 1989. Mosaic hybrid zones and the nature of species boundaries, pp. 111-133 *in* Otte, D. & Endler, JA. (eds) *Speciation and its Consequences*. Sinauer Associates, Sunderland, MA.

Hewitt, GM. 1988. Hybrid Zones—Natural laboratories for evolutionary studies. Trends in Ecology and Evolution 3(7): 158-167.

Husband, BC. 2004. The role of triploid hybrids in the evolutionary dynamics of mixed-ploidy populations. Biological Journal of the Linnean Society 82: 537-546.

Husband, BC. & Schemske, DW. 1998. Cytotype distribution at a diploid-tetraploid contact zone in *Chamerion (Epilobium) angustifolium* (Onagraceae). American Journal of Botany 85(12): 1688-1694.

Husband, BC. & Sabara, HA. 2003. Reproductive isolation between autotetraploids and their diploid progenitors in fireweed, *Chamerion angustifolium* (Onagraceae). New Phytologist 161: 703-713.

Ireland, RR. 1957. Biosystematics of *Erythronium albidum* and *E. mesochoreum*. M.A. dissertation, University of Kansas, Lawrence.

Kaul, RB. 1989. The Status of *Erythronium albidum* and *E. mesochoreum* (Liliaceae) in Nebraska. Transactions of the Nebraska Academy of Sciences 17: 71-79.

Knerr, EG. 1891. *Erythronium mesochoreum*. Transactions of the Kansas Academy of Sciences 13: 20-21.

Levin, DA. 1975. Minority cytotype exclusion in local plant populations. Taxon 24(1): 35-43.

Lopez, GA., Potts, BM., & Tilyard, PA. 2000. F_1 hybrid inviability in *Eucalyptus*: the case of *E. ovata* x *E. globulus*. Heredity 85: 242-250.

Lumaret, R. & Barrientos, E. 1990. Phylogenetic relationships and gene flow between sympatric diploid and tetraploid plants of *Dactylis glomerata* (Gramineae). Plant Systematics and Evolution 169: 81-96.

Mallet, J. 2005. Hybridization as an invasion of the genome. Trends in Ecology and Evolution 20(5): 229-237.

Mayr, E. 1942. *Systematics and the Origin of Species.* Columbia University Press, New York.

McClain, WE., Ebinger, JE., & Koelling, AC. 1999. Status and distribution of *Erythronium mesochoreum* Knerr (Liliaceae) in Illinois. Castanea 64(4): 346-349.

Muller, RN. 1979. Biomass accumulation and reproduction in *Erythronium albidum*. Bulletin of the Torrey Botanical Club 106 (4): 276-283.

Myers, JA., Vellend, M., Gardescu, S., & Marks, PL. 2004. Seed dispersal by white-tailed deer: implications for long-distance dispersal, invasion, and migration of plants in eastern North America. Oecologia 139: 35-44.

Nagy, ES. & Rice, KJ. 1997. Local adaptation in two subspecies of an annual plant: Implications for migration and gene flow. Evolution 51: 1079-1089.

Pascarella, JB. 2007. Mechanisms of prezygotic reproductive isolation between two sympatric species, *Gelsemium rankinii* and *G. sempervirens* (Gelsemiaceae), in the southeastern United States. American Journal of Botany 94(3): 468-476.

Peñaloza-Ramírez, JM., González-Rodríguez, A., Mendoza-Cuenca, L., Caron, H., Kremer, A., & Oyama, K. 2010. Interspecific gene flow in a multispecies oak hybrid zone in the Sierra Tarahumara of Mexico. Annals of Botany 105: 389-399.

Petit, C., Bretagnolle, F., & Felber, F. 1999. Evolutionary consequences of diploid-polyploid hybrid zones in wild species. Trends in Ecology and Evolution 14(8): 306-311.

Ramsey, J. & Schemske, DW. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annual Review of Ecology and Systematics 29: 467-501.

Ramsey, J., Bradshaw, HD., & Schemske, DW. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). Evolution 57(7): 1520-1534.

Rickett, HW. 1937. *Erythronium mesochoreum*. Rhodora 39: 101-105.

Rieseberg, LH. 1997. Hybrid origins of plant species. Annual Review of Ecology and Systematics 28: 359-389.

Rieseberg, LH. & Willis, JH. 2007. Plant speciation. Science 317: 910-914.

Robertson, KR. 1966. The genus *Erythronium* (Liliaceae) in Kansas. Annals of the Missouri Botanical Garden 53(2): 197-204.

Segraves, KA. & Thompson, JN. 1999. Plant polyploidy and pollination: floral traits and insect visits to diploid and tetraploid *Heuchera grossulariifolia*. Evolution 53 (4): 1114-1127.

Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture. Web Soil Survey. Available online at http://websoilsurvey.nrcs.usda.gov/. Accessed 04/09/2012.

Vasek, FC. & Sauer, RH. 1971. Seasonal progression of flowering in *Clarkia*. Ecology 52: 1038-1045.

Widmer, A., Lexer, C., & Cozzolino, S. 2009. Evolution of reproductive isolation in plants. Heredity 102: 31-38.

CHAPTER 3

EVALUATING MECHANISMS OF REPRODUCTIVE ISOLATION BETWEEN DIPLOID *ERYTHRONIUM MESOCHOREUM* AND ITS TETRAPLOID CONGENER *E. ALBIDUM* (LILIACEAE)

INTRODUCTION

The world's biodiversity is a direct product of the process of speciation, and modern inquiries into the nature of species originated with Darwin (Barrett *et al.* 1987). There are several species concepts, that is, methods of defining what constitutes a species (Table 1.1—Chapter 1). The biological species concept, however, directly addresses what many evolutionists consider the most salient question in this field (Coyne & Orr 2004): how are sexually reproducing taxa able to co-occur in sympatry without losing their genetic distinctiveness via gene flow?

The biological species concept states that species are formed when diverging populations become reproductively isolated via pre- and/or postzygotic barriers (Dobzhansky 1935, Mayr 1942). Several mechanisms may operate to create barriers to reproduction between diverging populations, thereby restricting gene flow (Coyne & Orr 2004, Sobel *et al.* 2010).

These barriers may arise incidentally as a byproduct of natural selection and genetic drift when diverging taxa become geographically isolated from one another (allopatric speciation; Coyne & Orr 1989, Knowlton *et al*. 1993, Xiang *et al.* 2000,

Crisp & Cook 2007). Alternatively, barriers may evolve in sympatry as a result of genome duplication (Otto & Whitton 2000, Keller & Gerhardt 2001), chromosomal rearrangements (Grant 1971, Rieseberg 1997), or selective pressures that favor divergence within a population (Giślason *et al.* 1999, Schliewen *et al.* 2001). Additionally, prezygotic barriers may arise to reinforce inchoate species boundaries when incipient species come into secondary contact with one another (Rundle & Schluter 1998, Hoskin *et al*. 2005).

Among plants, many isolating mechanisms can act to reduce gene flow between diverging taxa (Grant 1971) (Figure 3.1). Prezygotic barriers limit or prevent hybrid formation, and include habitat-based spatial isolation (Nagy & Rice 1997, Ramsey *et al.* 2003), flowering asynchrony (Husband & Schemske 2000, Pascarella 2007), and pollinator-based isolation (Grant 1949, Segraves & Thompson 1999). Postzygotic barriers cause hybrid mortality or reduce the fitness of hybrids, and include partial or complete hybrid inviability and hybrid sterility (Grundt *et al*. 2006, Burton & Husband 2000). Closely related plant species are generally reproductively isolated via a combination of multiple pre- and postzygotic mechanisms (Rieseberg & Willis 2007).

Many recent studies have focused intensely on evaluating the role that single reproductive barriers play in speciation and species boundary maintenance (Rieseberg *et al.* 1995, Bradshaw & Schemske 2003, Hentrich *et al.* 2010). Relatively few studies, however, have investigated the contribution of multiple barriers to reproductive isolation between closely related plant taxa (Rieseberg & Willis 2007, Widmer *et al*. 2009). Further, our understanding of the mechanisms underlying the

origin and maintenance of species boundaries between diploid-polyploid species pairs and cytotypes is not well developed (Husband & Sabara 2003). Understanding these processes is critical to the field of plant speciation, as it is estimated up to 80% of angiosperms have undergone genome duplication at some point in their evolutionary history (Soltis & Soltis 2009, Pires & Gaeta 2011), and that 15% of speciation events among angiosperms result from changes in ploidy (Wood *et al.* 2009).

Plant species that differ in ploidy are generally thought to be reproductively isolated via triploid block, a form of intrinsic postzygotic isolation wherein triploid hybrids are sterile or inviable as a result of multiple factors, including abnormal seed development and aneuploid gamete formation (Marks 1966, Coyne & Orr 2004). Although this is certainly the case for many diploid-tetraploid species and cytotype pairs (*i.e. Vaccinium* sp., Dweikat & Lyrene 1988; *Dactylis glomerata*, Lumaret & Barrientos 1990; *Arrhenatherum elatius*, Petit *et al.* 1999), recent evidence has demonstrated that triploid hybrids across many taxa retain some level of viability and fertility (Ramsey & Schemske 1998, Husband 2004). Furthermore, although genome duplication can confer nearly instantaneous reproductive isolation in the form of hybrid sterility and inviability, additional isolating barriers can arise between diploid-polyploid species pairs and cytotypes (Husband & Schemske 2000). The contributions of these additional barriers remain largely unstudied (Husband & Schemske 2000, Husband & Sabara 2003).

I investigated the role of multiple pre- and postzygotic barriers in the maintenance of species boundaries between a diploid-polyploid species pair in the

genus *Erythronium* (Liliaceae). *Erythronium albidum* Nutt. and *Erythronium mesochoreum* Knerr are perennial forbs native to eastern and central North America (Kaul 1989). These species are well-suited for investigations of multiple pre- and postzygotic reproductive barriers for many reasons. Although *E. albidum* is tetraploid (2n = 4x = 22) and *E. mesochoreum* is diploid (2n = 2x =22), they share the same base chromosome number (x = 11) and are likely sister species (Robertson 1966, Allen *et al.* 2003). The two species tend to inhabit different habitats (Churchill 1986). However, populations of *E. albidum* and *E. mesochoreum* abut throughout their respective ranges (Kaul 1989, McClain 1999). In addition, viable hybrid individuals have been documented at contact zones (see Chapter 2), indicating that reproductive isolation between these taxa may be incomplete and possibly of recent origin.

The goal of this study was to evaluate the contribution of multiple reproductive isolating mechanisms between *E. albidum* and *E. mesochoreum*. To do this, I **1)** assessed interspecific flowering asynchrony by measuring flowering overlap between *E. albidum* and *E. mesochoreum* across multiple spatial scales, **2)** investigated pollinator-based isolation by examining the degree to which the pollinator assemblages of the study species are non-overlapping, **3)** examined whether one or more post-pollination (late prezygotic, early postzygotic) barriers inhibits hybrid seed production using a hand-pollination experiment, and **4)** tested for discrepancy between conspecific and hybrid seed mass using seeds generated in the hand-pollination experiment, as a potential correlate of hybrid fitness. I demonstrate that although *E. albidum* and *E. mesochoreum* are physiologically

capable of producing hybrid seeds when hand-pollinated, they may be reproductively isolated from one another by virtue of a low degree of overlap in their pollinator assemblages.

MATERIALS AND METHODS

Study Species

E. albidum and *E. mesochoreum* are monoecious, perennial forbs that belong to the eastern North American clade of the genus *Erythronium* (Allen *et al*. 2003). *E. albidum*'s geographic range extends from the Midwest to the east coast of the United States, and from southern U.S. states north to Ontario, Canada. *E. mesochoreum'*s range is limited to central U.S. states (Figure 1.3—Chapter 1; Allen & Robertson 2002). *E. mesochoreum* primarily inhabits tallgrass prairie, although it is occasionally found in open woodlands, whereas *E. albidum* is predominantly found in wooded areas (Robertson 1966, Kaul 1989, McClain et al. 1999). Although these differences in habitat likely contribute to reproductive isolation between the taxa, populations of *E. mesochoreum* and *E. albidum* can abut at prairie-forest borders and can intergrade more widely in intermediate habitats such as oak savannahs (Kaul 1989, McClain 1999).

Though *E. mesochoreum* is diploid (2n = 2x = 22) and *E. albidum* is tetraploid (2n = 4x = 44), the two species share a base chromosome number of x = 11 (Robertson 1966), and both karyotype evidence and sequence data from the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA genes strongly suggest that *E. albidum* and *E. mesochoreum* are sister species (Allen *et al.*

2003). Further, I have confirmed that *E. albidum* and *E. mesochoreum* hybridize in at least one contact zone in southeast Nebraska (Chapter 2).

The plants of both species are monanthous, and they flower in early spring and senesce before the onset of summer. Identified pollinators of *E. mesochoreum* and *E. albidum* include the oligolectic solitary bee *Andrena erythronii* (Andrenidae; Michener & Rettenmeyer 1956) and the polylectic solitary bee *Andrena carlini* (Banks 1980), respectively.

Flowering Asynchrony

Interspecific asynchrony in flowering phenology was analyzed at two spatial scales—a larger scale analysis involving herbarium specimens from the Midwestern United States and a smaller scale study involving field observations at sites in eastern Nebraska.

Herbarium Study

In 2009-2011 I recorded phenological data from herbarium sheets of *E. albidum* and *E. mesochoreum* collected in Nebraska, Iowa, Kansas and Missouri (Table 3.1). Herbarium accessions were surveyed at eight institutions— the Field Museum (the Field Museum Herbarium; F), Harvard University (the Gray Herbarium; GH), Iowa State University (the Ada Hayden Herbarium, ISC; IA), the University of Kansas (the R.L. McGregor Herbarium; KANU), Kansas State University (the Kansas State University Herbarium; KSC), the Missouri Botanical Garden (the Missouri Botanical Garden Herbarium; MO), the University of Nebraska-Lincoln (the C.E. Bessey Herbarium; NEB), and the University of Missouri (the Dunn-Palmer Herbarium; UMO). For each accession, I recorded the number of plants on the

herbarium sheet, the collection locality, the collection date, and the phenological stage of each plant (vegetative, in bud, flowering, fruiting, unknown/damaged). Because *E. albidum* and *E. mesochoreum* are monanthous, each plant corresponded to only one phenological stage. Many herbarium sheets contained multiple plants, but a maximum of one plant per phenological stage per herbarium sheet was included in the analyses to avoid collection biases. In addition, groups of herbarium sheets collected at the same locality and exact date were treated as one sheet to account for any sampling locality bias. Geographic coordinates for each accession were determined from the collection locality data using Google Earth (Google Inc., 2011). Latitude and longitude were assigned based on the town or township nearest the collection locality. For accessions for which township data were not recorded, coordinates of the county seat for the county of collection were used. For each herbarium specimen in flower, I converted the calendar date of collection to the ordinal day. Thus, each flowering accession was assigned an ordinal flowering day (OFD).

Because OFD is likely strongly influenced by regional climatic conditions, I assigned a single mean annual temperature (MAT) and mean annual precipitation (MAP) value to each herbarium accession. Temperature and precipitation data were obtained from the High Plains Regional Climate Center (www.hprcc.unl.edu), which maintains records of climate data collected from weather stations in Midwestern states from the mid-1800s to the present. For each herbarium accession, I located the nearest weather station for which $\geq$ 90% of the daily average temperature and daily total precipitation values were available over $\geq$ 70 years.

There were a small number of accessions for which a weather station was not located within 50 km of the collection locality. These accessions were eliminated from all subsequent analyses.

I calculated a single MAT and MAP value, averaged across several decades of climate data, for each herbarium accession. First, monthly MAT and MAP values for a single year were averaged to calculate yearly MAT and MAP values. Months for which $\geq$ 4 data points (days) were missing were excluded from the yearly average. Then, each year's value was averaged across the entire period of record (through 2011) for MAT and MAP values, respectively. The average time period over which MAT was calculated was 89 years. Similarly MAP was calculated, on average, over 88 years. Single year averages for which $\geq$ 1 monthly average was missing were not included in the calculations of the overall MAT or MAP means.

I then created a general linear model that investigated the effects of species, collection year, MAT and MAP on OFD, using a Type III test of effects. The full model tested the main effects of each of these terms, as well as all 2-, 3- and 4-way interactions. Beginning with the highest-order interaction term, I removed non-significant terms from the model one at a time based on F-tests (Crawley 2007). Non-significant terms were pooled into error. All analyses were carried out in R (version 2.11.1; R Development Core Team 2010).

Field Study

In spring 2010 and 2011 I established a total of 20 study quadrats (1 m^2) in seven populations of *E. mesochoreum* and *E. albidum* (Table 3.2; Figure 1.6—Chapter 1). Each quadrat contained plants of only one study species. I labeled all

mature (2-3 leaved) *Erythronium* plants within each quadrat as they emerged using numbered aluminum tags. Single-leaved *Erythronium* plants have never been observed to flower and thus were not marked. In 2011 one site (Red Cedar Recreation Area) did not have a sufficient density of flowering *E. albidum* plants to track flowering progression in small quadrats; therefore, flowering phenology of individual plants was tracked in the same manner across a larger (~ 0.2 ha) area. I visited each study quadrat 1-3 times per week during the growing season to label newly emerged plants and to categorize the phenological status of each plant as vegetative, budding, flowering, or fruiting until all tagged plants in the quadrat had senesced. Because *Erythronium* flowers open and close rapidly as weather conditions fluctuate, late-stage budding plants were marked as "flowering".

After the data were collected, I calculated the proportion of mature plants in flower in each quadrat during each calendar week of the growing season. If multiple records were made during one week, the proportion of plants in flower was averaged across observations in that week. I then used Pianka's overlap index (Pianka 1974; Equation 3.1) to quantify the mean interspecific flowering overlap (O_{jk}) for every pairwise *E. albidum—E. mesochoreum* quadrat comparison for both 2010 and 2011.

To assess whether there was significant flowering asynchrony I created a null model, modified from Ashton *et al.* (1988), to compare the observed O_{jk} values with a distribution of overlap values generated under the null hypothesis of no interspecific flowering asynchrony. The null distribution was created by randomly shifting the position of peak flowering intensity for each quadrat within the

observed *Erythronium* flowering season, while preserving the shape of the flowering intensity distributions of each quadrat. For each randomization, an O_{jk} value was calculated, and this process was iterated 1000 times. The 2010 and 2011 flowering seasons were modeled separately. Significant asynchrony was determined using a one-tailed test if the observed O_{jk} fell at or below the fifth percentile of the null distribution of O_{jk} values from the model.

Pollinator-Based Isolation

To assess pollinator-based isolation, I measured pollinator assemblage overlap by capturing insects as they were visiting the flowers of *E. albidum* and *E. mesochoreum* at three sites in eastern Nebraska— Bur Oak Wildlife Management Area (WMA), Madigan Prairie, and Red Cedar Recreation Area (Figure 1.6—Chapter 1). Insect capture occurred over four collecting trips in 2010 and five collecting trips in 2011 (a total of 9 hours of collecting time). To limit the number of non-pollinating insects collected, I only captured insects that were observed to be in contact with the reproductive structures of *E. albidum* or *E. mesochoreum* flowers. The captured insects were immediately killed in ethyl acetate kill jars and pinned within 36 hours of collection. The pinned specimens were sent to the USDA-ARS Systematic Entomology Laboratory for identification to species.

Insect specimens were grouped among sites and study years to create a list of all insects observed to visit *E. albidum* and *E. mesochoreum* flowers. I used Pianka's overlap index (Pianka 1974; Equation 3.1) to quantify overlap (O_{jk}) between the pollinator assemblages of the study plant species. To assess whether there was significant non-overlap in the pollinator assemblages, I created a null

model using the 'vegan' package in R (Oksanen *et al.* 2011). Working under the null hypothesis that all insects were equally available to both *E. albidum* and *E. mesochoreum* flowers, I randomly assigned individual insects to either *E. albidum* or *E. mesochoreum*. I kept constant both the total number of insects collected on each plant species, as well as the total number of individuals belonging to each insect species, so that the permutations would accurately reflect the relative abundances of insect species as well as the total abundance of insects captured on each plant species. I calculated O_{jk} for each of 1000 iterations of the model to create a distribution of O_{jk} values against which to compare the observed O_{jk} value. Probabilities were calculated based on a 1-tailed test, and I considered there to be significant non-overlap in the pollinator assemblages of *E. albidum* and *E. mesochoreum* if the observed value fell at or below the fifth percentile of the null distribution of O_{jk} values.

Post-Pollination Barriers

To test whether post-pollination barriers limit hybrid seed production, I performed a total of 281 hand-pollinations of *E. albidum* and *E. mesochoreum* flowers at Madigan Prairie (*E. mesochoreum* pollen recipients) and Bur Oak WMA (*E. albidum* pollen recipients) in 2010 and 2011 (Figure 1.6—Chapter 1). All pollen recipients were emasculated prior to floral anthesis, and I performed both conspecific and heterospecific crosses (Table 3.3). I used emasculated, non-pollinated plants as a negative control. Of the 30 negative control plants, one *E. albidum* individual set seed. Previous studies have not recorded apomictic seed production for *E. albidum* (Schemske *et al.* 1978, Banks 1980). This indicates that

the plant's exclosure may have been breached by a pollinator, or that apomixis is possible for this species but is extremely uncommon.

Because the flowers of *E. albidum* and *E. mesochoreum* can be long-lived and I had not previously determined the timing or duration of stigma receptivity, I hand-pollinated each study plant twice: 48-72 hours after emasculation of the pollen recipient and 48-72 hours after the first application. I thoroughly coated the stigmas of the study plants with pollen to minimize the chances of pollen limitation. In addition, I collected naturally occurring *E. albidum* and *E. mesochoreum* fruits at Bur Oak WMA and Madigan Prairie against which to test the efficacy of the hand-pollinations.

As the fruits from the hand-pollinations were maturing, I applied Tanglefoot or petroleum jelly around the perimeter of the exclosures to discourage seed predation and seed dispersal by insects. I collected the fruits at maturity and stored them in coin envelopes until they dehisced naturally. Upon dehiscence, I counted the number of ovules per fruit, calculated seed set (number of developed seeds per fruit/total number of ovules per fruit) and measured the average seed mass per fruit. All statistical analyses for the hand-pollination experiment were carried out in R (version 2.11.1).

Seed set data from the hand-pollination experiment were divided into two sets for hypothesis testing. First, data from intraspecific crosses of *E. albidum* and *E. mesochoreum* were used to test whether the study species were self-compatible, whether outcrossing affected seed set, and whether I applied sufficient pollen by comparing hand-pollinated fruits to wild-collected fruits. The second dataset was

used to test for differences in seed set between conspecific (intra-population) and heterospecific crosses, and to assess whether *E. albidum* and *E. mesochoreum* fruits differed in seed set, regardless of pollen donor.

For both datasets, I used a similar approach to analysis in which seed set was modeled as a binomial process, $m_j \sim \text{Binomial}(N_j, p_j)$, where m_j represents the proportion of seed set per fruit, N_j represents the number of ovules in a fruit, and p_j represents the probability that an ovule will set seed. I analyzed the seed set datasets using generalized linear models (McCullagh & Nelder 1989). Because the models' residual deviance values were larger than the residual degrees of freedom, I fit the overdispersion parameter for all models (Crawley 2007). Eight individuals that received pollen treatments subsequently failed to set fruit. These accessions were treated as having zero seed set.

For the first seed set dataset, seed set was modeled as a function of treatment, year, and the treatment x year interaction for *E. mesochoreum* and *E. albidum*, separately. "Treatment" was a categorical variable with four levels: "self" represented fruits that were self-pollinated; "conspecific, intra-population" represented fruits that received conspecific pollen from within the pollen recipient's population; "conspecific, outcross" represented fruits that received conspecific pollen from an outside population, and "wild-collected" represented fruits that were collected from the pollen recipient's population. "Year" was a categorical variable with two levels (2010, 2011) that represented the two years during which the experiment was performed. When a significant interaction between treatment and year occurred, the effect of pollination treatment on seed set was analyzed

separately for each year. Non-significant factors were pooled into error, and *a priori* linear contrasts were used to assess differences in seed set among treatment groups.

For the second seed set dataset, seed set was modeled as a function of cross type, maternal species, year, and all two- and three-way interactions of these terms. "Cross type" was a categorical variable with two levels, "conspecific" and "heterospecific", that represented whether the plant received conspecific (intra-population) or heterospecific pollen, respectively. "Maternal species" was a categorical variable with two levels, *E. albidum* and *E. mesochoreum*, that represented the species identity of the pollen recipient. The year term was a categorical variable with two levels, 2010 and 2011. Due to a significant three-way interaction, the effects of cross type, maternal species, and the cross type x maternal species interaction on seed set were analyzed for 2010 and 2011, separately. *A priori* linear contrasts were used to investigate differences in seed set among specific treatment groups.

I tested for differences in ovule number per fruit between *E. albidum* and *E. mesochoreum* to ascertain whether interspecific differences in seed set would translate into differences in seed production. The ovule number dataset consisted of all intact fruits collected from Madigan Prairie and Bur Oak WMA. I created a generalized linear model with a Poisson error distribution to examine the effects of maternal species, year, and the maternal species x year interaction on ovule number per fruit for *E. albidum* and *E. mesochoreum* (see above for factor definitions and levels). When a non-significant interaction term was identified, the maternal

species x year interaction term was pooled into error, and the model was re-run without this term.

Hybrid and Parental Species Seed Mass

Because reduced hybrid seed mass could confer lower fitness to hybrids, compared to the parental species, I tested for differences in average seed mass between fruits from conspecific (intra-population) and heterospecific crosses. I also tested whether *E. albidum* and *E. mesochoreum* fruits differed in average seed mass, regardless of pollen donor. The seed mass data were first log-transformed to conform to the normality assumption. A general linear model was used to model the effects of maternal species, cross type, and year, and all possible two- and three-way interactions, on the log-transformed average seed mass (see above for factor definitions and levels). Due to a significant interaction involving the "year" term, the effects of cross type and maternal species were analyzed separately for the 2010 and 2011 data.

RESULTS

Flowering Asynchrony

Herbarium Study

The herbarium dataset encompassed 254 flowering plants collected from 1873-2007. For *E. albidum* accessions (collected from 1873-2004), flowering ranged from March 24—May 27. Flowering for *E. mesochoreum* individuals (collected from 1881-2007) ranged from March 3—May 2.

All four-, three- and two-way interactions, as well as the main effect of MAP, were eliminated from the full model, based on their lack of significance ($P > 0.05$). The final model consisted only of the main effects of species, collection year, and MAT, which all had statistically significant effects on the ordinal flowering day (OFD) (Figure 3.2; Table 3.4). Taking into account collection year and MAT, *E. mesochoreum* flowered on average 7.11 ± 1.32 days earlier than *E. albidum* (model estimate ± 1 standard error) (Figure 3.2A). Both species demonstrated similar decreases in OFD from 1873 to 2007, flowering an average of 0.047 ± 0.020 days earlier per year (model estimate ± 1 standard error) (Figure 3.2B). *E. albidum* and *E. mesochoreum* responded to MAT similarly as well, with a 2.14 ± 0.29 day decrease in OFD per degree increase in MAT (model estimate ± 1 standard error).

Field Study

Flowering phenology of *E. albidum* and *E. mesochoreum* in Nebraska exhibited substantial overlap in both 2010 and 2011. Mean (± 1 standard error) O_{jk} for 2010 was 0.861 ± 0.048, while mean O_{jk} for 2011 was 0.605 ± 0.045 (Figure 3.3). Based on the null model, there was no statistically significant flowering asynchrony between *E. mesochoreum* and *E. albidum* in either 2010 or 2011 ($P_{2010} = 0.649$; $P_{2011} = 0.559$; Figure 3.4).

Pollinator-Based Isolation

A total of 69 insects representing 14 species were captured visiting *E. albidum* or *E. mesochoreum* flowers in 2010 and 2011 (Table 3.5). The two most frequent floral visitors were both solitary bees belonging to the genus *Andrena* (Andrenidae). *Andrena carlini* was the most frequent visitor of *E. albidum* flowers,

whereas *Andrena erythronii* was the most frequent visitor of *E. mesochoreum* flowers. Overall, three species—*Andrena carlini, Osmia pumila* (Megachilidae), and *Ceratina calcarata* (Apidae)—were captured on both *E. albidum* and *E. mesochoreum* flowers, comprising 43% of all visits across both *Erythronium* species. Of these species, *Andrena carlini* was the most frequent visitor (35 % of all visits) but was captured nearly four times more often on *E. albidum* than on *E. mesochoreum*.

The observed interspecific overlap (O_{jk}) between the pollinator assemblages of *E. albidum* and *E. mesochoreum* was 0.24. The null model yielded a distribution of O_{jk} values ranging from 0.59-0.98. Because the observed O_{jk} value fell below the lowest O_{jk} value in the null distribution, I concluded that there was significant non-overlap between the pollinator assemblages of *E. albidum* and *E. mesochoreum* ($P < 0.001$).

Post-Pollination Barriers

Self Compatibility, Outcrossing, and Effectiveness of Hand-Pollinations on Seed Set

For *E. albidum*, the effect of pollination treatment on seed set differed between years, so data for each year were analyzed separately (significant interaction term; Table 3.6). *E. albidum* is self-compatible (Figure 3.5). In 2010, seed set values for selfed *E. albidum* individuals and conspecific (intra-population) hand-pollinations were not statistically distinguishable from each other ($P = 0.093$; Figure 3.5A), although in 2011, selfed *E. albidum* individuals set significantly more seed than fruits from conspecific (intra-population) hand-pollinations ($P = 0.003$; Figure 3.5B).

Outcrossing did not consistently increase seed set for *E. albidum*—in 2010, individuals that received pollen from an outside population did not have significantly higher seed set than fruits resulting from intra-population pollinations ($P = 0.185$; Figure 3.5A). However, in 2011 outcrossed individuals had significantly higher seed set ($P = 0.002$; Figure 3.5B).

For *E. mesochoreum*, the effect of pollination treatment on seed set did not differ between years (non-significant interaction term; Table 3.6). Pollination treatment significantly affected seed set for *E. mesochoreum* ($P < 0.001$; Table 3.6), although collection year did not ($P = 0.301$; Table 3.6). *E. mesochoreum* is self-compatible, but seed set was significantly lower for selfed individuals than for hand-pollinated (intra- and inter-population pollinations) and wild collected fruits ($P < 0.001$; Figure 3.6). Outcrossing significantly decreased seed set, compared to seed set resulting from intra-population pollinations ($P = 0.023$; Figure 3.6).

There was no evidence that pollen limitation affected seed set for hand-pollinated *E. albidum* or *E. mesochoreum* plants. Seed set for wild-collected *E. albidum* fruits from the study population was significantly lower than seed set for conspecific (intra- and inter-population) hand-pollinations in 2010 ($P = 0.025$; $P = 0.011$, respectively; Figure 3.5A). In 2011 seed set for wild-collected *E. albidum* fruits was significantly lower than that of outcrossed plants ($P = 0.018$) but was not significantly different from that of conspecific, intra-population hand-pollinations ($P = 0.434$; Figure 3.5B). Seed set from wild-collected *E. mesochoreum* fruits from the study population did not significantly differ from seed set resulting from intra- or inter-population hand-pollinations ($P = 0.180$; $P = 0.330$, respectively; Figure 3.6).

Conspecific vs Heterospecific Seed Set

Heterospecific hand-pollinations demonstrated that *E. albidum* and *E. mesochoreum* are capable of producing hybrid seeds (Figure 3.7). However, the effects of cross type on seed set were dependent upon year and the identity of the maternal species ($P = 0.045$), so the 2010 and 2011 analyses were conducted separately. In both 2010 and 2011, fruits from conspecific hand-pollinations set more seed than fruits from heterospecific hand-pollinations ($P_{2010} < 0.001$, $P_{2011} < 0.001$, Figure 3.7; Table 3.7). Seed set was reduced by 46% in 2010 and 43% in 2011 when *E. albidum* fruits were pollinated with heterospecific, versus conspecific, pollen. Seed set was reduced by 29% in 2010 and 47% in 2011 when *E. mesochoreum* fruits were pollinated with heterospecific pollen.

Additionally, *E. mesochoreum* fruits set significantly more seed than *E. albidum* fruits, regardless of pollen donor, in both study years ($P_{2010} < 0.001$, $P_{2011} < 0.001$, Figure 3.7, Table 3.7). In 2010, the effects of cross type on seed set did not depend on maternal species identity (non-significant interaction term; Table 3.7). This interaction was significant, however, in 2011 (Table 3.7) and resulted from the fact that there was no significant drop in seed set when *E. albidum* was pollinated with heterospecific (vs conspecific) pollen in 2011 ($P = 0.109$; Figure 3.7B), whereas the drop in seedset from heterospecific pollination of *E. mesochoreum* was significant in 2011 ($P < 0.001$; Figure 3.7B).

Number of Ovules Per Fruit

There was no significant interaction between maternal species and year on the number of ovules per fruit ($P = 0.118$). Both maternal species identity and the

year in which the study was performed significantly affected ovule number. *E. mesochoreum* fruits had significantly more ovules than *E. albidum* fruits ($P = 0.048$), and ovule number was significantly greater in 2010, versus 2011 ($P = 0.029$) (Table 3.8). Despite the statistical significance of these differences, the actual difference in ovule number between the study species and the study years was slight. *E. mesochoreum* had an average ($\pm$ 1 standard error) of 34.56 $\pm$ 0.82 ovules per fruit, whereas *E. albidum* fruits had, on average, 33.22 $\pm$ 0.51 ovules. Similarly, fruits collected in 2010 had an average ($\pm$ 1 standard error) of 34.45 $\pm$ 0.65 ovules, while 2011 fruits had 33.01 $\pm$ 0.64 ovules.

Hybrid and Parental Species Seed Mass

The effect of maternal species identity on average seed mass depended on the year in which the study was performed ($P = 0.006$), so the data were subset by year. There was no evidence that average mass was lower for hybrid seeds than for seeds of the parental species. In 2010, average seed mass did not vary significantly between cross types or maternal species (Table 3.9, Figure 3.8A). In 2011, there were no significant differences in average seed mass for seeds resulting from conspecific and hybrid crosses (Table 3.9). However, in 2011, seeds for which *E. albidum* was the pollen recipient had significantly greater average mass than seeds for which *E. mesochoreum* was the pollen recipient, regardless of pollen donor identity ($P < 0.001$, Table 3.9, Figure 3.8B). Interactions between cross type and the maternal species' identity were not significant in either 2010 or 2011 (Table 3.9).

DISCUSSION

Research that addresses multiple components of reproductive isolation between closely related taxa is critical to improving our understanding of speciation, yet few studies address this theme (Widmer *et al.* 2009). My study assessed the contributions of multiple pre- and postzygotic barriers to the maintenance of species boundaries between two trout lilies (*Erythronium* spp.). Overall, I found that the barriers I investigated were not uniform in strength. Flowering asynchrony was not a consistently strong reproductive barrier. Further, hybrid seeds were produced when I hand-pollinated *Erythronium* flowers, though seed set was reduced by approximately 29-47%, depending on the direction of the cross. However, interspecific pollen transfer is likely uncommon at contact zones, as the pollinator assemblages of *E. albidum* and *E. mesochoreum* are highly non-overlapping. Therefore, pollinator-based isolation is likely a strong barrier to hybridization between *E. albidum* and *E. mesochoreum.*

Many of the barriers I assessed were asymmetric, restricting hybridization in one specific direction over the other. These asymmetric barriers may influence patterns of hybridization and gene flow between *E. albidum* and *E. mesochoreum*. Despite the attention generally given to triploid block, my study highlights the importance of considering prezygotic barriers in investigations of species formation and species perpetuation between taxa that differ in ploidy.

Flowering Asynchrony

Although there was some discrepancy in interspecific flowering phenology across a broad geographic scale, flowering asynchrony is likely not a strong enough barrier to prevent interspecific pollen transfer at *E. albidum*-*E. mesochoreum* contact zones. My herbarium study indicated that *E. mesochoreum* flowers, on average, several days earlier than *E. albidum* across Midwestern states, after controlling for patterns of temperature and precipitation. Even though *E. mesochoreum* flowered earlier across a broad geographic scale, there was still substantial interspecific overlap in the ranges of *E. mesochoreum* and *E. albidum* flowering dates recorded from herbarium accessions.

Despite interspecific differences in flowering phenology on a broad geographic scale, there was no significant flowering asynchrony between *E. albidum* and *E. mesochoreum* in my eastern Nebraska study plots. However, in 2011 some of the *E. albidum* plots initiated flowering later than the *E. mesochoreum* plots, and flowering in *E. albidum* plots persisted after all flowering in *E. mesochoreum* plots was finished (Figure 3.3B).

Flowering phenology differences have been documented between polyploids and their diploid progenitors (Bretagnolle & Thompson 1996, Ramsey & Schemske 1998), although it is not known whether tetraploid *E. albidum* is derived from diploid *E. mesochoreum* (see below). Differences in flowering time between polyploids and their diploid progenitors may be the direct result of an increased genome copy number (Husband & Schemske 2000). Flowering phenology differences between *E. albidum* and *E. mesochoreum* may also result from variations

in environmental conditions, such as soil temperature, that are concordant with their differing habitats (or microhabitats, in the case of contact zones). I tracked soil temperature approximately 6 cm belowground at many of the eastern Nebraska sites where I conducted flowering observations, and I found that soil temperatures were similar among sites. However, prairie sites (*i.e.* Madigan and Te Amo Prairies) tended to have slightly higher soil temperatures (Figure 3.9), which could promote the earlier initiation of *E. mesochoreum* flowering. Overall, I do not know the mechanisms that underlie the observed slightly earlier flowering for *E. mesochoreum*, but this phenomenon is likely due to a combination of genetic effects and differences in environmental conditions between *E. albidum* and *E. mesochoreum* habitats.

Interannual variation in *E. albidum* and *E. mesochoreum* flowering phenology should cause the strength of this reproductive barrier to vary from year to year. Individual *E. albidum* and *E. mesochoreum* flowers can persist for several days, so a difference in flowering initiation of several days may limit, but not eliminate gene flow between these species in most years. Flowers of both species are also protandrous, which certainly influences patterns of interspecific pollen deposition, given their differences in flowering time. The observed differences in flowering phenology between *E. albidum* and *E. mesochoreum* favor the transfer of pollen from *E. albidum* anthers to *E. mesochoreum* stigmas, as *E. mesochoreum* is the earlier-flowering species.

Pollinator-Based Isolation

My study indicates that the pollinator assemblages of *E. albidum* and *E. mesochoreum* are distinct in species composition, suggesting that gene flow via interspecific pollen transfer through shared pollinators is likely to be infrequent. Although three out of 14 insect species were captured visiting both plant species, only one of these insect species, *Andrena carlini*, was an abundant floral visitor. Even though it visited both plant species, *Andrena carlini* was captured four times more frequently on *E. albidum* than *E. mesochoreum* flowers. This lack of overlap between *E. albidum* and *E. mesochoreum*'s pollinator assemblages likely poses a major reproductive barrier between the two species, as it would be expected to strongly limit the extent of interspecific pollen transfer.

My analysis of pollinator assemblage overlap between *E. albidum* and *E. mesochoreum*, however, includes some caveats. Because I grouped the pollinator data among sites, my assessment of overlap assumes that there were no site-specific differences in the available pollinator assemblages, and two of the three study sites contained only one *Erythronium* species. Some insect species, such as *Andrena erythronii*, were captured at only one site, so they may have been absent or scarce at the other sites. On the other hand, many species, including *Andrena carlini*, were captured at $\geq$ 2 study sites. In addition, when I restricted the null model's randomization procedure to include only insects that were found at both an *E. albidum* and an *E. mesochoreum* site, I still found highly significant non-overlap in the pollinator assemblages (data not shown), suggesting that grouping data across sites did not affect my conclusions.

Shared pollinator species may display fidelity to either *E. albidum* or *E. mesochoreum*, which would reduce the proportion of interspecific pollinator flights, the true indicator of pollinator-based reproductive isolation. Pollinator behavior has been demonstrated to cause reproductive isolation between plants, including diploid-polyploid cytotypes, in previous studies (Grant 1949, Husband & Schemske 2000). Although my null model does not explicitly take into account pollinator behavior, if interspecific pollinator flights were frequent, I would expect to have captured more pollinators on both *Erythronium* species. Further, during my collecting trips it did not appear that pollinators demonstrated fidelity to either *E. albidum* or *E. mesochoreum* (K. Roccaforte, personal observation). I therefore conclude that low interspecific pollen transfer, due to a lack of abundance of shared pollinators, likely plays a key role in maintaining species boundaries between *E. albidum* and *E. mesochoreum*.

Post-Pollination Barriers

The results of my hand-pollination experiment indicate that, while hybrid seeds were produced, one or more reproductive barriers acted to reduce hybrid seed set. Multiple late prezygotic and early postzygotic barriers may be responsible for the observed reduction in hybrid seed production. Heterospecific pollen can suffer reduced siring ability, compared to conspecific pollen (Harder *et al.* 1993, Rieseberg *et al.* 1995, Williams *et. al* 1999, Swanson *et al.* 2004, Rahmé *et al.* 2009). In addition, hybrid embryos may be aborted at higher rates, due to genetic incompatibilities between the parental species (Drake 1981, Abbo & Ladizinsky 1991).

Differences in seed set between *E. albidum* and *E. mesochoreum* that were independent of whether the cross was conspecific or heterospecific have some interesting implications for reproductive isolation between these species. *E. mesochoreum* fruits, regardless of pollen donor, set significantly greater seed than *E. albidum* fruits in both 2010 and 2011. Therefore, despite the fact that heterospecific pollen applications reduced seed set more consistently for *E. mesochoreum* than for *E. albidum* pollen recipients in both study years, *E. mesochoreum* fruits still had higher hybrid seed set in both years. Furthermore, because there were only small differences in ovule number between *E. albidum* and *E. mesochoreum* fruits, greater *E. mesochoreum* seed set translated into greater hybrid seed production for *E. mesochoreum* fruits, versus *E. albidum* fruits. Interestingly, seed set for hybrids where *E. mesochoreum* was the pollen recipient was actually higher than seed set resulting from conspecific *E. albidum* crosses.

The factors underlying the discrepancy in seed set between *E. albidum* and *E. mesochoreum* remain unclear, but these differences may be a product of the relative frequencies of asexual, versus sexual reproduction, for each species. *E. albidum* populations are comprised primarily of single-leaved vegetatively reproducing or non-reproductive plants (Muller 1979). Flowering plants comprise only a small proportion of *E. albidum* populations, and seedling recruitment for *E. albidum* is low, whereas juvenile plant recruitment from corms is high (Muller 1979). Little is known about the reproductive biology of *E. mesochoreum,* but it has been observed that flowering plants occur more commonly in *E. mesochoreum* populations, compared to *E. albidum* populations (Ireland 1957, Kaul 1989).

These life history differences indicate that sexual reproduction may be a more important component of *E. mesochoreum* propagation than it is for *E. albidum*. Therefore, *E. mesochoreum* may allocate more biomass to sexual reproduction than does *E. albidum*, which may be reflected by overall higher levels of seed set for *E. mesochoreum*. Previous observations have confirmed that natural levels of seed production are generally higher for *E. mesochoreum* than for *E. albidum* (Churchill 1986).

Hybrid and Parental Species Seed Mass

I did not find any significant differences in average seed mass between hybrid and parental species seeds, indicating that hybrid seeds likely do not suffer from reduced fitness due to a lack of seed provisioning. However, germination and seedling survival for hybrids and the parental species were unable to be assessed (see below).

Asymmetry of Reproductive Barriers

Taking into account all of the reproductive barriers I investigated, I expect the production of EM x EA hybrids to be favored over EA x EM hybrids because of the combined effects of protandry, slightly earlier flowering by *E. mesochoreum*, and greater average seed set by *E. mesochoreum*. Asymmetry in individual reproductive barriers has been observed in other plant taxa (Ramsey *et al.* 2003, Kay 2006, Rahmé *et al.* 2009), including diploid-polyploid species pairs and cytotypes (Williams *et al.* 1999, Husband *et al.* 2002). Asymmetrical reproductive isolation has the potential to influence the direction in which gene flow occurs between taxa that are not completely reproductively isolated from one another. If hybrids

produced between *E. albidum* and *E. mesochoreum* are fertile and capable of backcrossing, this may favor the transfer of *E. albidum* genes into the *E. mesochoreum* genome, but limit the transfer of *E. mesochoreum* genes into the *E. albidum* genome.

Additional Potential Reproductive Barriers

Although both *E. mesochoreum* and *E. albidum* are physiologically capable of producing hybrid seeds when hand-pollinated, few hybrid individuals were identified at the contact zones I surveyed (see Chapter 2). This suggests that pollinators are not transferring pollen interspecifically, as my study indicates. In addition, barriers that were not included in my studies could confer reproductive isolation between *E. albidum* and *E. mesochoreum*. It should be noted that habitat-based spatial isolation likely plays a role in maintaining reproductive isolation between the study species, though it was not explicitly examined in my studies. Throughout their geographic ranges, *E. albidum* and *E. mesochoreum* tend to inhabit different types of habitats (Kaul 1989). Further, my systematic sampling for hybrid individuals (Chapter 2) revealed that both *E. albidum* and *E. mesochoreum* aggregate with conspecifics at contact zones (Figure 2.3—Chapter 2), although it remains unclear whether this occurs in response to microhabitat differences. Hybrid inviability and sterility, due to triploid block, may also confer reproductive isolation between *E. albidum* and *E. mesochoreum.* My attempts to germinate *E. albidum*, *E. mesochoreum*, and hybrid seeds using the methodology outlined by Baskin & Baskin (1985) were unsuccessful, so I was unable to examine differences in germination or seedling mortality among hybrids and the parental species.

The Role of Prezygotic Barriers in Polyploid Speciation

The presence of prezygotic barriers to hybridization between *E. albidum* and *E. mesochoreum* emphasizes the importance of considering these barriers when investigating the origin and maintenance of species boundaries between closely related plant taxa that differ in ploidy. Although immediate and substantial postzygotic barriers can arise between plants that differ in ploidy due to triploid block, prezygotic barriers such as habitat-based spatial isolation, flowering asynchrony and pollinator-based isolation have been shown to occur between plants that differ in cytotype (Husband & Schemske 2000, Ramsey 2011, Glennon *et al.* in press). Early-acting barriers may play an important role in the establishment of polyploid populations by reducing the frequency of intercytotype mating in mixed populations of diploids and polyploids (see below), and thus facilitate polyploid speciation (Fowler & Levin 1984).

The Origins of E. albidum

Although the evolutionary origin of *E. albidum* is not known, a reasonable hypothesis is that tetraploid *E. albidum* arose from diploid *E. mesochoreum* via autopolyploid speciation. This hypothesis is supported by the fact that, aside from the clonal endemic *E. propullans*, *E. albidum* and *E. mesochoreum* are the only *Erythronium* species that have x = 11 as a base chromosome number. In addition, ITS sequence data indicate that they are likely sister taxa (Allen *et al.* 2003). Molecular data across numerous polyploid taxa have demonstrated that polyploid species often arise via multiple genome duplication events within their diploid progenitors' range (reviewed by Soltis & Soltis 1993; see also Segraves *et al.* 1999,

Albach 2007). Therefore, *E. albidum* may be derived from multiple, independent *E. mesochoreum* genome duplications. Although there are many mechanisms of polyploid formation, gametic non-reduction has been shown to be among the most common (Ramsey & Schemske 1998), and the union of unreduced (diploid) *E. mesochoreum* gametes across one or more populations may have initially given rise to *E. albidum*.

After polyploid individuals are formed, the most pertinent immediate challenge to the establishment of polyploid species is minority cytotype exclusion (Levin 1975). This occurs in mixed-ploidy populations when (initially rare) polyploids suffer a reproductive disadvantage, as the majority of their mating opportunities come from (more abundant) diploids and result in low-fitness hybrids. Minority cytotype exclusion can lead to the extirpation of polyploids within diploid populations and thus prevent polyploid species from establishing, as polyploids are literally "bred out" of existence (Husband & Sabara 2003). Fortunately, there are many ways for polyploids to overcome minority cytotype exclusion.

Within small populations of diploids, polyploids can establish due to demographic stochasticity (Parisod *et al.* 2010). Recurrent polyploid formation can provide a constant influx of polyploids that can also aid polyploid establishment (Coyne & Orr 2004, Parisod *et al.* 2010). Perhaps more importantly, polyploid establishment can be fostered by traits that limit the extent of inter-cytotype mating. These traits include phenotypic divergence, increased competitive abilities of polyploids, and reproductive assurance (*e.g.* self-compatibility, vegetative

reproduction) (Otto 2007, Parisod *et al.* 2010, Oswald & Nuismer 2011). The relative importance of these mechanisms for polyploid speciation has been the subject of investigation for several decades.

Phenotypic divergence of polyploids may result because genome duplication can alter the cytological, biochemical, genetic, and developmental traits of plants (Levin 1983). However, polyploidy's immediate effects on plant morphology are often small, and there do not appear to be any universal, predictable modifications to plants due to genome duplication (Levin 1983, Otto 2007, Soltis *et al.* 2007, Parisod *et al.* 2010). Nevertheless, morphological changes that may promote polyploid establishment and speciation have been observed across many taxa (Levin 1983). Changes in flower phenology and morphology as a result of genome duplication may promote ecological divergence and, thus, assortative mating (Levin 1983, Bretagnolle & Thompson 1996, Ramsey & Schemske 1998, Husband & Schemske 2000). Increased drought tolerance and increased growth rates may allow polyploids to outcompete diploids (Levin 1983, Flagel & Wendel 2009). Further, the development of traits that promote reproductive assurance (*e.g.* breakdowns in self-incompatibility and an increasing propensity for vegetative reproduction after genome duplication; Levin 1983, Ramsey & Schemske 2002) can also promote the persistence of polyploid populations.

Interestingly, *E. albidum* utilizes vegetative reproduction more often than does *E. mesochoreum*. In addition, although both species are self-compatible, selfing did not reduce seed set for *E. albidum*, as it did for *E. mesochoreum* (Figures 3.5 and 3.6). If these traits arose in *E. albidum* as a result of genome duplication and were

present during the initial stages of speciation, they would certainly have acted to help *E. albidum* overcome minority cytotype exclusion and establish within *E. mesochoreum* populations. Assortative mating among *E. albidum* individuals may have also initially been fostered by prezygotic barriers, such as habitat-based spatial isolation or a slightly later flowering time, that developed as a result of genome duplication and reduced the frequency of inter-cytotype pollinations.

Despite the current importance of pollinator-based isolation in the maintenance of reproductive isolation between *E. albidum* and *E. mesochoreum* as indicated by my studies, it remains unclear whether pollinator-based isolation played a predominant role in the initial establishment of *E. albidum*. One of the challenges of studying speciation is that it is often difficult to determine whether reproductive barriers that are currently strong are the ones that were responsible for speciation (Coyne & Orr 2004). Shifts in pollinator assemblages resulting from changes in flower phenology and morphology have been documented in mixed-cytotype populations, but it is difficult to ascertain whether these changes played a role in the speciation process or occurred after speciation was complete (Coyne & Orr 2004).

Andrena carlini, the most frequent floral visitor to *E. albidum* and an infrequent visitor to *E. mesochoreum*, is a polylectic species that may have been present at the time of *E. albidum*'s speciation. If so, then it is possible that divergence in microhabitat preference or small alterations to flowering phenology resulting from genome duplication may have promoted *E. albidum's* transition to *Andrena carlini* as a pollinator. Assuming that, as is the case now, *Andrena carlini*

was not a frequent pollinator of *E. mesochoreum*, this shift in pollination services might have provided sufficient protection against minority cytotype exclusion to allow the persistence of the polyploid individuals that would ultimately become *E. albidum.* However, detailed morphometric studies of *Erythronium* flowers, which have not yet been conducted, are required to determine whether there is a better morphological match between *Andrena carlini* and *E. albidum*, versus *E. mesochoreum*.

At several of my study sites, *E. albidum* was the first spring ephemeral that I observed in flower, and the timing of *Andrena carlini* emergence appeared to be tightly correlated with *E. albidum* flowering. Because many environmental factors influence the phenology of flowering and insect emergence, it seems more likely that the traits underlying this close coordination between *E. albidum* flowering and *Andrena carlini* emergence evolved after the initial phases of *E. albidum* speciation, rather than as a direct result of genome duplication.

Conclusions

My study adds to the growing body of evidence indicating that the strength of various forms of reproductive isolation between closely related plants may not be uniform, and that the strength of different components of reproductive isolation between taxa can be asymmetrical. Therefore, my research emphasizes the importance of considering multiple forms of reproductive isolation in assessments of plant species boundaries. Further, I found that a prezygotic barrier, pollinator-based isolation, likely confers strong reproductive isolation between my study species. Thus, despite the acknowledged importance of triploid block in most

diploid-polyploid plant systems, future research in the field of plant speciation and the maintenance of species boundaries should address the contributions of prezygotic reproductive barriers.

EQUATIONS

$$O_{jk} = \frac{\sum_{i=1}^{n} p_{ij} p_{ik}}{\sqrt{\sum_{i=1}^{n} p_{ij}^2 \sum_{i=1}^{n} p_{ik}^2}}$$

Equation 3.1: Pianka's overlap index (O_{jk}) ranges from zero (no overlap) to one (complete overlap). For the flowering phenology study, *p* refers to the proportion of plants in flower in a given quadrat, *j* and *k* refer to *E. albidum* and *E. mesochoreum* study quadrats, respectively, *i* refers to a time block of one calendar week, and *n* refers to the total number of calendar weeks. For the pollinator assemblage overlap study, *p* refers to the proportion of the total insects found *E. albidum* or *E. mesochoreum,* respectively, that belong to insect species *i. E. albidum* and *E. mesochoreum* are represented by *j* and *k*, and *n* refers to the total number of insect species found on *E. albidum* and *E. mesochoreum.*

TABLES

Table 3.1: Total number of *E. albidum* and *E. mesochoreum* herbarium accessions analyzed, partitioned by state of origin.

State	*E. albidum*	*E. mesochoreum*
Iowa	14	3
Kansas	61	91
Missouri	7	20
Nebraska	42	16

Table 3.2: Location of study quadrats used in the flowering overlap study in spring 2010 and 2011 (EA = *E. albidum*; EM = *E. mesochoreum*). The bold numbers indicate the total number of unique quadrats (1 quadrat each at Te Amo Prairie and Bur Oak WMA was tracked for both years), and the numbers in parentheses represent the number of quadrats tracked at each site in 2010 and 2011, respectively.

Site	Location	Latitude	Longitude	Species	Quadrats
Bauermeister Prairie	Douglas Co., NE	41.215° N	96.166° W	EM	**4** (2,2)
Madigan Prairie	Saunders Co., NE	41.169° N	96.881° W	EM	**4** (1,3)
Te Amo Prairie	Douglas Co., NE	41.192° N	96.208° W	EM	**2** (1,2)
Bur Oak WMA	Seward Co., NE	40.896° N	97.000° W	EA	**3** (1,3)
Pioneers Park	Lancaster Co., NE	40.772° N	96.772° W	EA	**2** (1,1)
Red Cedar Recreation Area	Saunders Co., NE	41.169° N	96.880° W	EM, EA	**3** (0,3)
Papio Creek railbed	Sarpy Co., NE	41.149° N	96.002° W	EA	**2** (0,2)

Table 3.3: Sample sizes for hand-pollinated flowers and wild collected fruits of *E. albidum* (EA) and *E. mesochoreum* (EM) in 2010 and 2011. Bold numbers represent total sample sizes, and the numbers in parentheses represent sample sizes for 2010 and 2011, respectively.

Cross	Pollinations	
EA control	**15**	(0,15)
EA x self	**18**	(4,14)
EA x EA (intra-population)	**44**	(30,14)
EA x EA (inter-population)	**14**	(4,10)
EA x EM	**55**	(40,15)
EA wild-collected	**61**	(26,15)
EM control	**15**	(0,15)
EM x self	**19**	(5,14)
EM x EM (intra-population)	**51**	(38,13)
EM x EM (inter-population)	**22**	(8,14)
EM x EA	**58**	(43,15)
EM wild-collected	**54**	(9,15)

Table 3.4: Analysis of variance table from a general linear model investigating the effects of mean annual temperature, species, and collection year on ordinal flowering day (OFD) for *Erythronium* herbarium accessions collected in Nebraska, Iowa, Missouri, and Kansas.

	D.f.	S.S.	M.S.	F	*P*
Mean annual temperature (MAT)	1	9625.40	9625.40	94.46	< 0.001
Species	1	2977.00	2977.00	29.21	< 0.001
Year	1	556.70	556.70	5.46	0.02
Residuals	250	25475.40	101.90		

Table 3.5: Identity and abundance of insects captured while visiting flowers of *E. albidum* and *E. mesochoreum*, with family names in parentheses. Insect collections were made in 2010 and 2011 at Bur Oak Wildlife Management Area (Seward Co., NE), Madigan Prairie (Saunders Co., NE), and Red Cedar Recreation Area (Saunders Co., NE). Insect identification was provided by the USDA-ARS Systematic Entomology Laboratory.

***Species* (Family)**	***E. albidum***	***E. mesochoreum***
Andrena algida (Andrenidae)	4	0
Andrena carlini (Andrenidae)	19	5
Andrena erythronii (Andrenidae)	0	20
Apis mellifera (Apidae)	3	0
Bombus bimaculatus (Apidae)	1	0
Bombylius major (Bombyliidae)	3	0
Ceratina calcarata (Apidae)	2	1
Halictus rubicundus (Halictidae)	0	1
Lasioglossum cressonii (Halictidae)	1	0
Lasioglossum forbesii (Halictidae)	0	1
Nomada luteoloides (Apidae)	2	0
Osmia lignaria (Megachilidae)	2	0
Osmia pumila (Megachilidae)	2	1
unknown Dipteran (Anthomyiidae)	0	1

Table 3.6: Analysis of deviance table for the generalized linear models assessing the effects of pollination treatment, study year, and the treatment x year interaction on seedset from *E. albidum* and *E. mesochoreum* fruits resulting from intraspecific crosses. The residual degrees of freedom and residual deviance values for the intercept-only model are listed in the table to compare model fit as additional terms are added.

	D.f.	Deviance	Residual D.f.	Residual Deviance	F	*P*
E. albidum						
Intercept			114	457.27		
Treatment	3	54.45	111	402.83	5.79	0.001
Year	1	1.70	110	401.13	0.54	0.463
Treatment x Year	3	37.59	107	363.54	4.00	0.010
E. mesochoreum						
Intercept			107	856.77		
Treatment	3	312.09	104	544.68	21.68	< 0.001
Year	1	5.19	103	539.49	1.08	0.301
Treatment x Year	3	29.91	100	509.58	2.08	0.108

Table 3.7: Analysis of deviance table from the generalized linear model testing the effects of cross type, maternal species, and the cross type x maternal species interaction on *E. albidum* and *E. mesochoreum* seed set in 2010 and 2011. The residual degrees of freedom and residual deviance values for the intercept-only model are listed in the table to compare model fit as additional terms are added.

	D.f.	Deviance	Residual D.f.	Residual Deviance	F	*P*
2010						
Intercept			138	1342.13		
Cross type	1	199.90	137	1142.23	58.33	< 0.001
Maternal species	1	635.69	136	506.54	185.48	< 0.001
Cross type x Maternal species	1	0.03	135	506.52	0.0076	0.931
2011						
Intercept			56	685.77		
Cross type	1	85.91	55	599.86	33.86	< 0.001
Maternal species	1	437.93	54	161.93	172.60	< 0.001
Cross type x Maternal species	1	18.54	53	143.40	7.31	0.009

Table 3.8: Analysis of deviance table from the generalized linear model investigating the effects of maternal species and year on ovule number per fruit for *E. albidum* and *E. mesochoreum* fruits. *P* values were calculated using a Chi-square distribution. The residual degrees of freedom and residual deviance values for the intercept-only model are listed in the table to compare model fit as additional terms are added.

	D.f.	Deviance	Residual D.f.	Residual Deviance	*P*
Intercept			291	520.42	
Maternal species	1	3.91	290	516.51	0.048
Year	1	4.74	289	511.77	0.029

Table 3.9: Analysis of variance table from the general linear model testing the effects of cross type, maternal species, and the cross type x maternal species interaction on the log-transformed average seed mass of *E. albidum* and *E. mesochoreum* fruits in 2010 and 2011.

	D.f.	S.S.	M.S.	F	*P*
2010					
Cross type	1	0.04	0.04	0.30	0.584
Maternal species	1	0.06	0.06	0.39	0.532
Cross type x Maternal species	1	0.14	-0.14	0.96	0.329
Residuals	136	20.06	0.15		
2011					
Cross type	1	0.01	0.01	0.06	0.804
Maternal species	1	2.73	2.73	29.73	< 0.001
Cross type x Maternal species	1	0.12	0.12	1.27	0.265
Residuals	49	4.51	0.09		

FIGURES

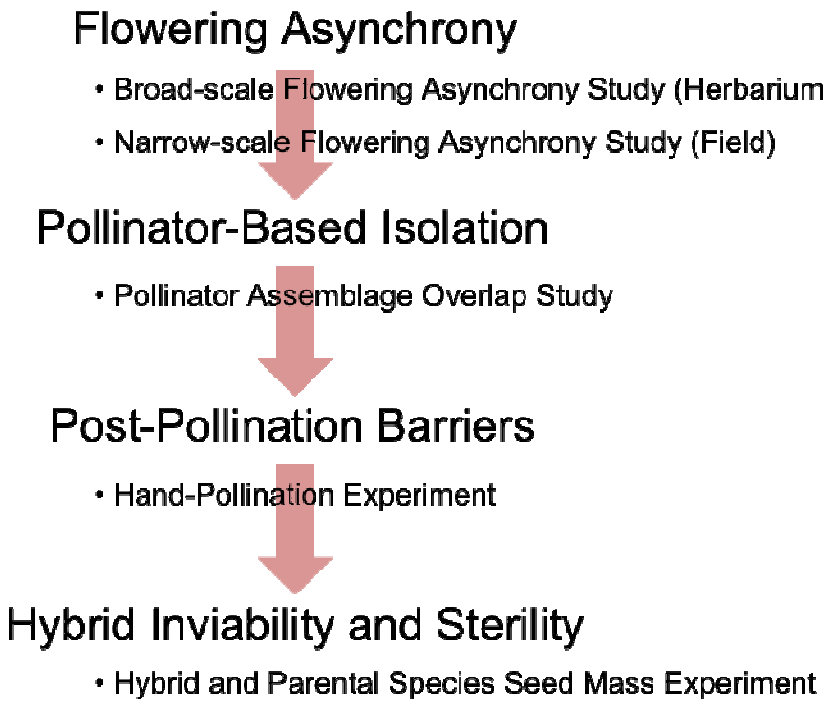

Figure 3.1: Flowchart of selected reproductive barriers that may act to prevent fertile hybrid formation and, thus, gene flow between angiosperm taxa. Reproductive barriers are listed in large-type, and the studies that I performed to address the contributions of each barrier to the maintenance of species boundaries between *E. albidum* and *E. mesochoreum* are listed as bullet-points below.

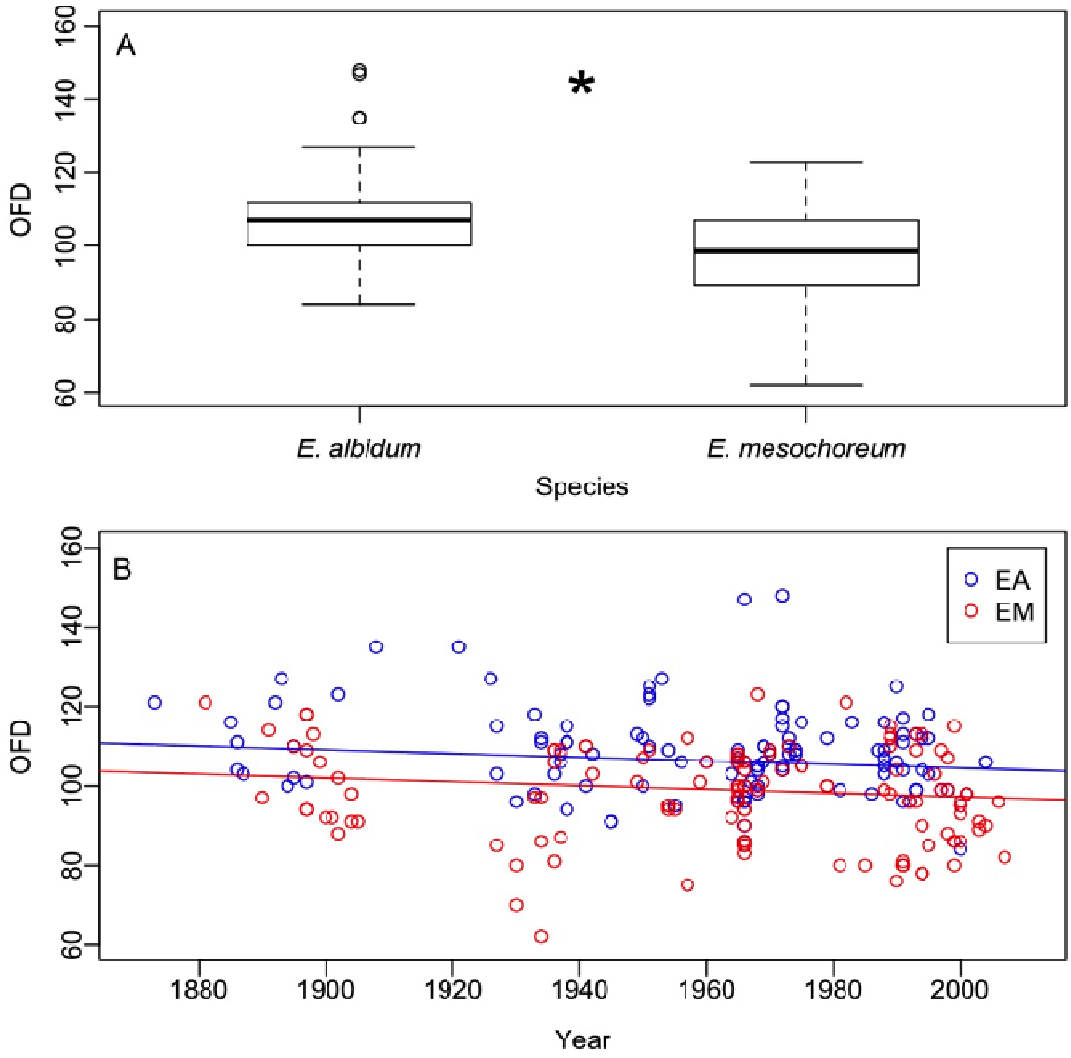

Figure 3.2: **A.** Ordinal flowering day (OFD) for 254 *E. albidum* and *E. mesochoreum* herbarium accessions collected in Nebraska, Iowa, Kansas, and Missouri from 1873-2007. The asterisk indicates a significant difference in OFD between *E. albidum* and *E. mesochoreum* accessions at $P \leq 0.05$, taking into account collection year and mean annual temperature (MAT). **B.** OFD plotted against the year of collection for *E. albidum* (EA) and *E. mesochoreum* (EM) herbarium accessions. Regression lines for *E. albidum* and *E. mesochoreum*'s OFD versus year are plotted based on values from the linear model, using the mean MAT value from the climate dataset.

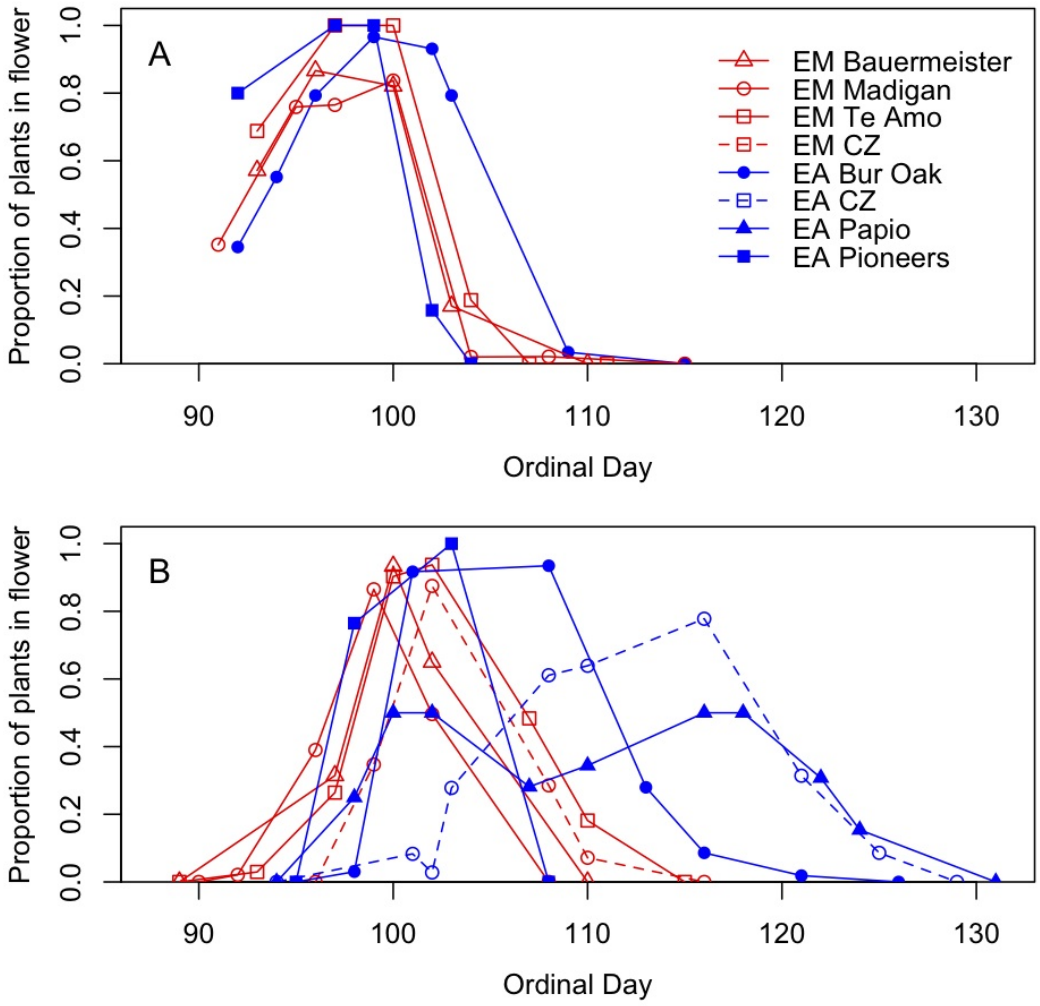

Figure 3.3: Flowering phenology overlap for study populations of *Erythronium mesochoreum* (EM, red lines) and *Erythronium albidum* (EA, blue lines). **A.** Spring 2010; **B.** Spring 2011. Dashed lines indicate data recorded at a zone of species contact. Mean interspecific overlap (± 1 standard error) was 0.861 ± 0.048 in 2010 and 0.605 ± 0.045 in 2011.

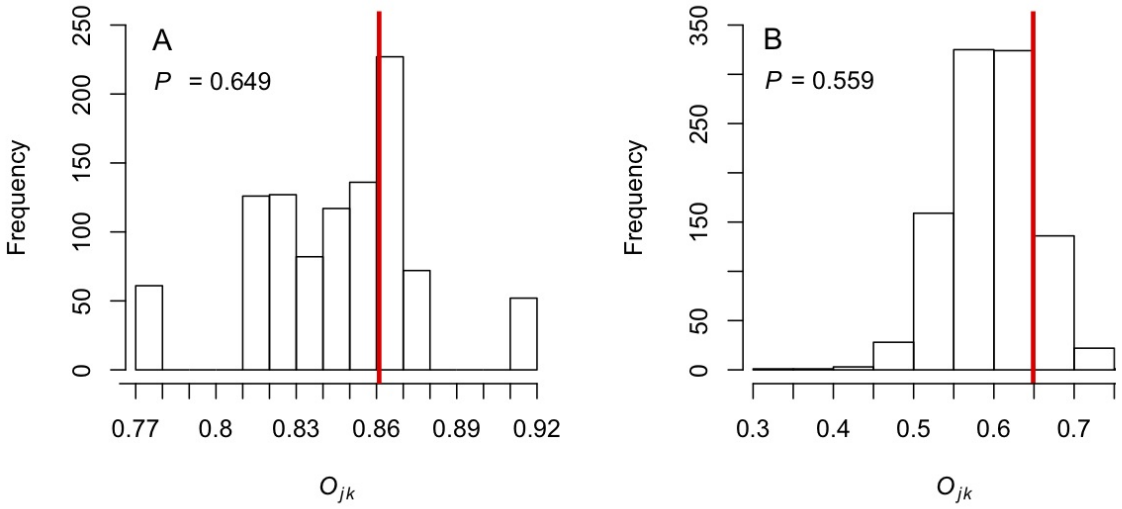

Figure 3.4: Histograms of the distribution of O_{jk} values created by the null models for **A.** 2010, and **B.** 2011. The red lines indicate the position of the observed O_{jk} value within each distribution. Mean interspecific overlap ($\pm$ 1 standard error) was 0.861 $\pm$ 0.048 in 2010 and 0.605 $\pm$ 0.045 in 2011. $P \leq 0.05$ indicates significant asynchrony in flowering time between *Erythronium albidum* and *Erythronium mesochoreum*.

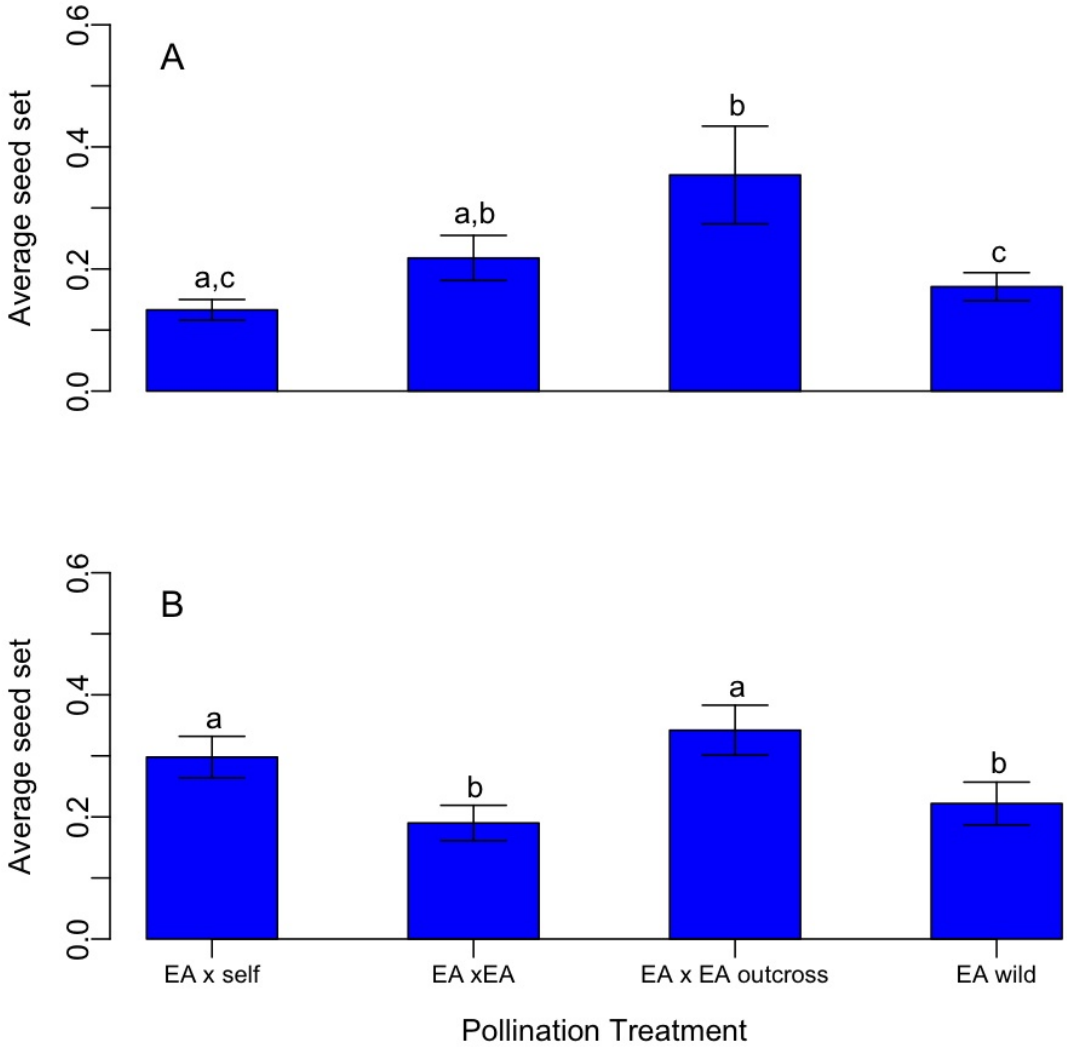

Figure 3.5: Average seed set ± 1 standard error for *Erythronium albidum* (EA) fruits resulting from self-pollination (EA x self), intraspecific, intra-population pollination (EA x EA), intraspecific pollination using pollen from outside of the study population (EA x EA outcross) and wild collected fruits (EA wild) in **A.** 2010, and **B.** 2011. Differing letters above the bars for each sub-figure represent significant differences

in seed set at $P \leq 0.05$, based on planned comparisons after a significant main effect of pollination treatment, in a one-way analysis of deviance.

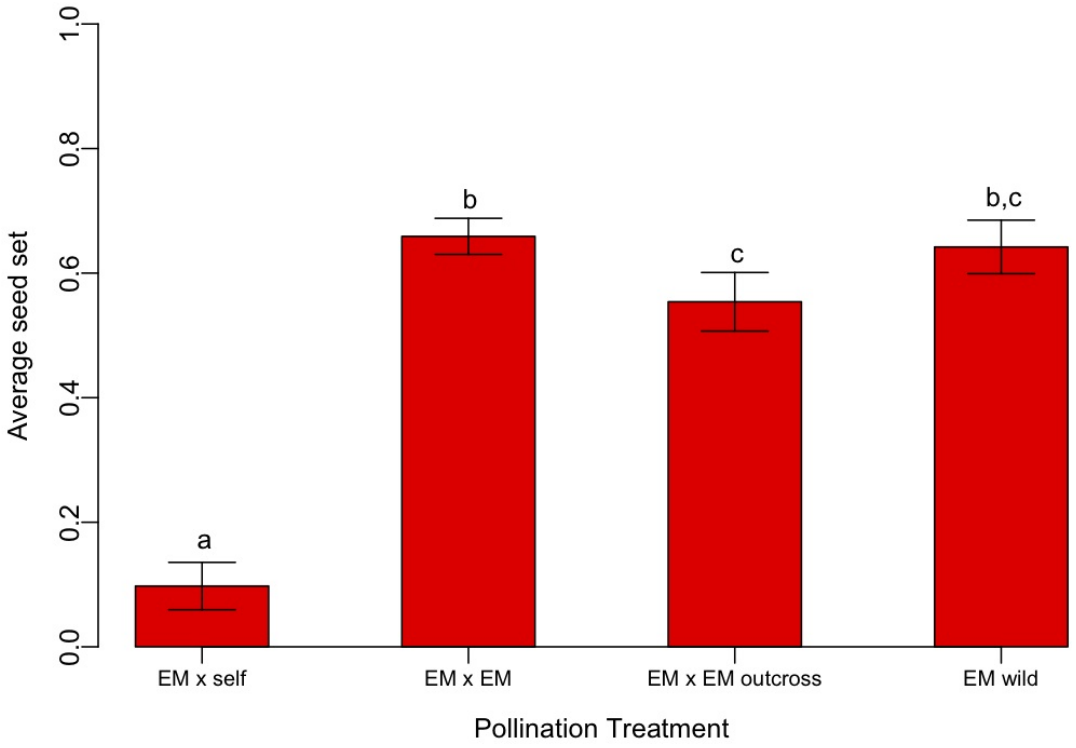

Figure 3.6: Average seed set ± 1 standard error for *Erythronium mesochoreum* (EM) fruits resulting from self-pollination (EM x self), intraspecific, intra-population pollination (EM x EM), intraspecific pollination using pollen from outside of the study population (EM x EM outcross) and wild collected fruits (EM wild) in 2010 and 2011. The effect of year was not significant, so data were pooled across years. Differing letters above the bars represent significant differences in seed set at $P \leq 0.05$, based on planned comparisons after a significant main effect of pollination treatment, in a one-way analysis of deviance.

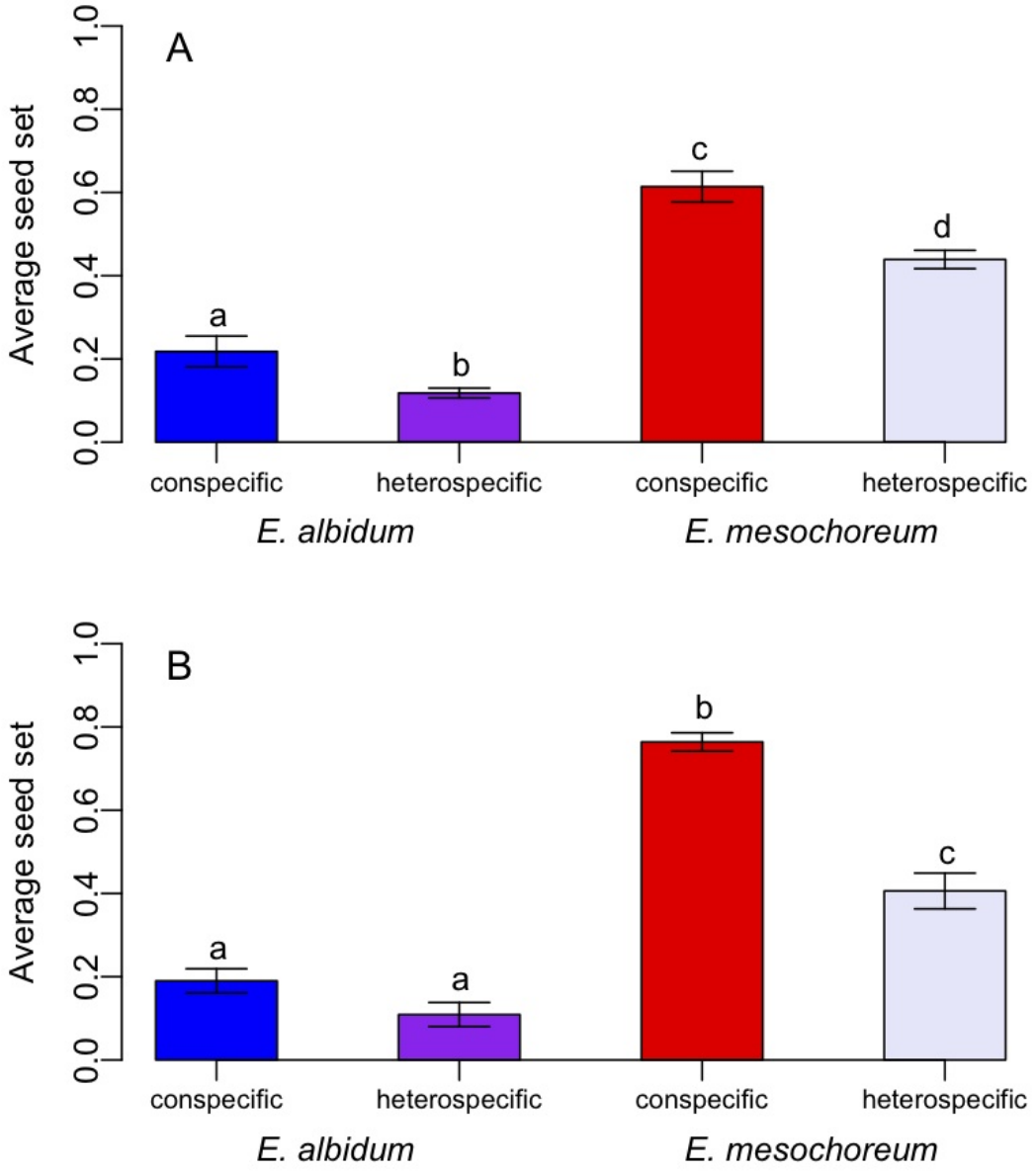

Figure 3.7: Average seed set ± 1 standard error from conspecific and heterospecific pollinations of *Erythronium albidum* (EA) and *Erythronium mesochoreum* (EM) in **A**. 2010, and **B**. 2011. Differing letters above the bars for each sub-figure represent significant differences in seed set at $P \leq 0.05$, based on planned comparisons after a significant main effect of cross type, in a one-way analysis of deviance.

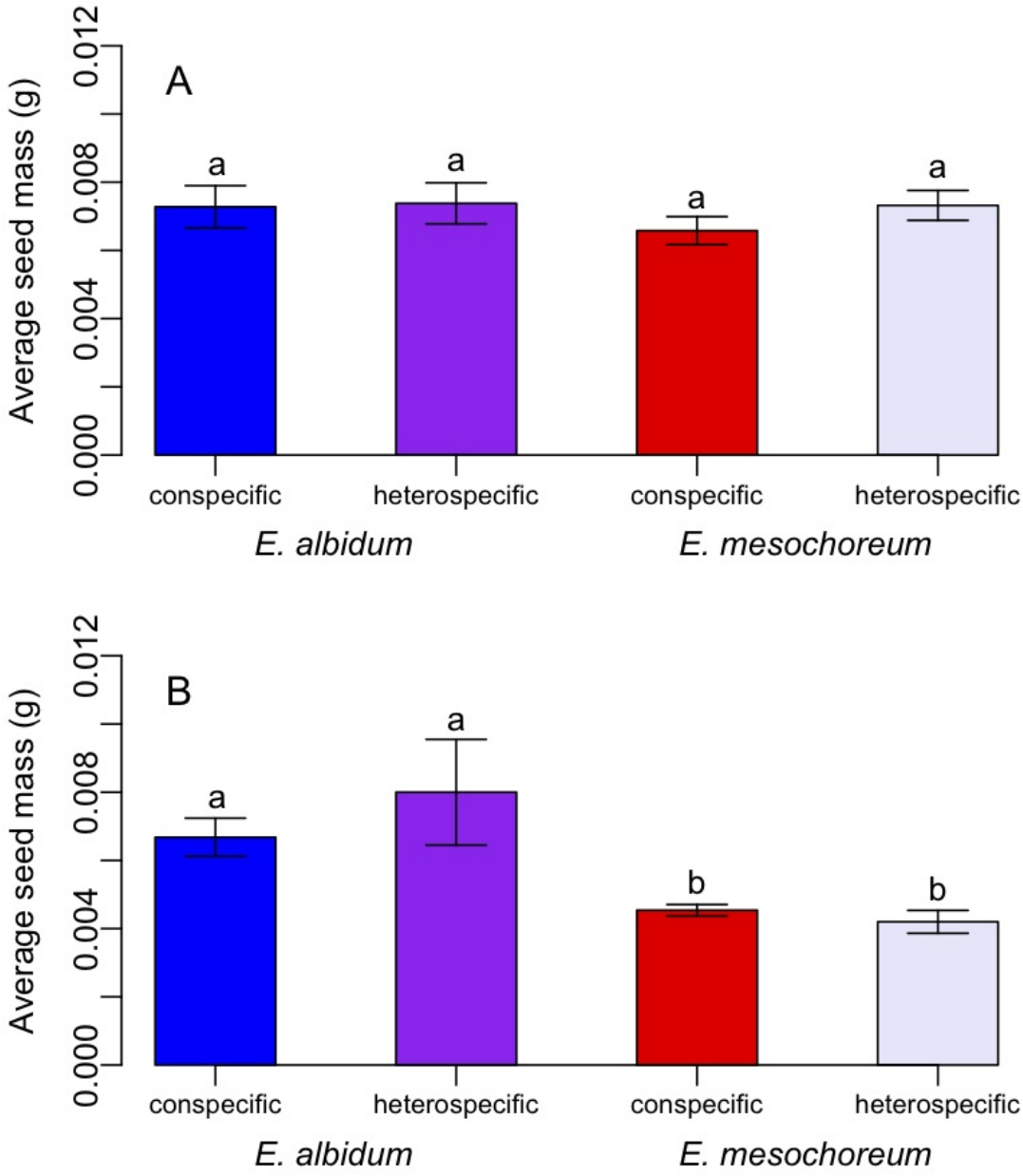

Figure 3.8: Average seed mass per fruit (g) ± 1 standard error for conspecific and heterospecific crosses of *Erythronium albidum* and *Erythronium mesochoreum* in, **A.** 2010, and **B.** 2011. Differing letters above the bars represent significant differences in average seed mass at $P \leq 0.05$, using a one-way analysis of variance, after a significant main effect of maternal species.

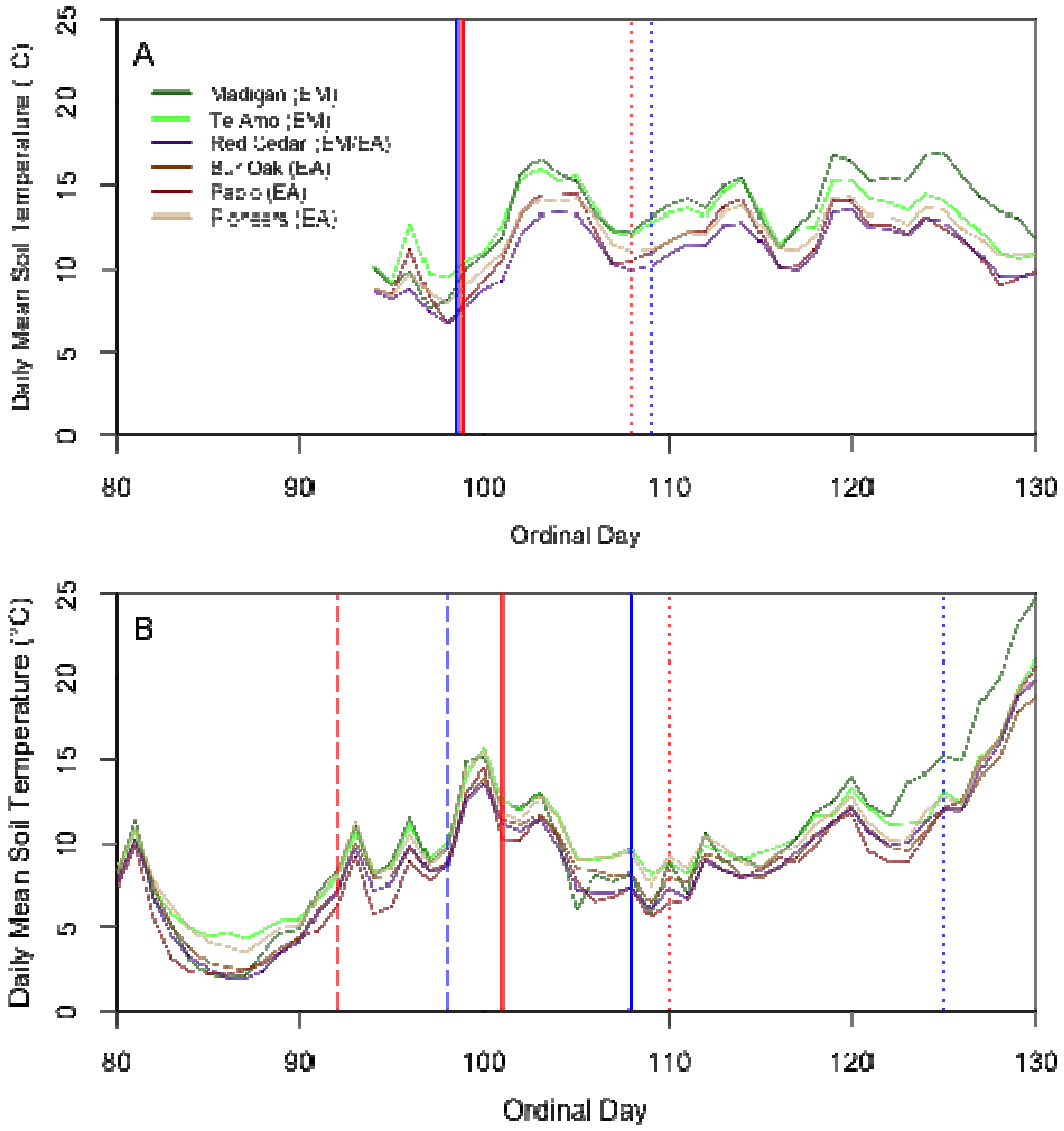

Figure 3.9: Daily mean soil temperature recorded approximately 6 cm belowground at *Erythronium albidum* (EA) and *Erythronium mesochoreum* (EM) eastern Nebraska field sites from March 21st to May 10th in, **A.** 2010 and, **B.** 2011. Vertical lines represent the day of first recorded flowering (long-dashed lines), the approximate peak of flowering (solid lines) and the last recorded day of flowering (dotted lines) for *E. albidum* (blue lines) and *E. mesochoreum* (red lines). Vertical lines were positioned based on flowering phenology data from my field survey of

eastern Nebraska study plots. In 2010, study plots were established after the onset of flowering. Therefore, no lines indicating the first recorded day of flowering are displayed in 2010.

REFERENCES

Abbo, S. & Ladizinsky, G. 1991. Anatomical aspects of hybrid embryo abortion in the genus *Lens* L. Botanical Gazette 152(3): 316-320.

Albach, DC. 2007. Amplified fragment length polymorphisms and sequence data in the phylogenetic analysis of polyploids: multiple origins of *Veronica cymbalaria* (Plantaginaceae). New Phytologist 176: 481-498.

Allen, GA. & Robertson, KR. 2002. Erythronium. pp. 153-164 *in* Flora of North America Editorial Committee (eds.) *Flora of North America, Vol. 26.* Oxford University Press, New York.

Allen, GA., Soltis, DE., & Soltis, PS. 2003. Phylogeny and biogeography of *Erythronium* (Liliaceae) inferred from chloroplast *matK* and nuclear rDNA ITS sequences. Systematic Botany 28(3): 512-523.

Ashton, PS., Givnish, TJ., & Appanah, S. 1988. Staggered flowering in the Dipterocarpaceae: New insights into floral induction and the evolution of mast fruiting in the aseasonal tropics. The American Naturalist 132(1): 44-66.

Banks, JA. 1980. The reproductive biology of *Erythronium propullans* Gray and sympatric populations of *E. albidum* Nutt. (Liliaceae). Bulletin of the Torrey Botanical Club 107(2): 181-188.

Barrett, PH., Gautrey, PJ., Herbert, S., Kohn, D., & Smith, S. 1987. *Charles Darwin's Notebooks, 1836-1844: Geology, Transmutation of Species, Metaphysical Enquiries.* Cornell University Press, Ithaca, NY.

Baskin, JM. & Baskin, CC. 1985. Seed germination ecophysiology of the woodland spring geophyte *Erythronium albidum*. Botanical Gazette 146(1): 130-136.

Bradshaw, HD. & Schemske, DW. 2003. Allele substitution at a flower color locus produces a pollinator shift in monkeyflowers. Nature 426: 176-178.

Bretagnolle, F. & Thompson, JD. 1996. An experimental study of ecological differences in winter growth between sympatric diploid and autotetraploid *Dactylis glomerata*. Journal of Ecology 84(3): 343-351.

Burton, TL. & Husband, BC. 2000. Fitness differences among diploids, tetraploids, and their triploid progeny in *Chamerion angustifolium*: Mechanisms of inviability and implications for polyploid evolution. Evolution 54: 1182-1191.

Churchill, SP. 1986. Liliaceae Juss., the Lily Family. pp. 1241-1258 *in* Great Plains Flora Association (eds.) *Flora of the Great Plains*. University Press of Kansas, Lawrence, KS.

Coyne, JA. & Orr, HA. 1989. Patterns of speciation in *Drosophila*. Evolution 43: 362-381.

Coyne, JA. & Orr, HA. 2004. *Speciation*. Sinauer Associates, Sunderland, MA.

Crawley, MJ. 2007. *The R Book.* John Wiley & Sons Ltd, West Sussex, England.

Crisp, MD. & Cook, LG. 2007. A congruent molecular signature of vicariance across multiple plant lineages. Molecular Phylogenetics and Evolution 43: 1106-1117.

Dobzhansky, T. 1935. A critique of the species concept in biology. Philosophy of Science 2: 344-355.

Drake, DW. 1981. Reproductive success of two *Eucalyptus* hybrid populations. II. Comparison of predispersal seed parameters. Australian Journal of Botany 29(1): 37-48.

Dweikat, IM. & Lyrene, PM. 1988. Production and viability of unreduced gametes in triploid interspecific blueberry hybrids. Theoretical and Applied Genetics 76: 555-559.

Flagel, LE. & Wendel, JF. 2009. Gene duplication and evolutionary novelty in plants. New Phytologist 183: 557-564.

Fowler, N. & Levin, DA. 1984. Ecological constraints on the establishment of a novel polyploid in competition with its diploid progenitor. The American Naturalist 124: 703-711.

Giślason, D., Ferguson, MM., Skúlason, & Snorasson, SS. 1999. Rapid and coupled phenotypic differentiation in Icelandic Arctic char (*Salvelinus alpinus*). Canadian Journal of Fisheries and Aquatic Sciences 56: 2229-2234.

Glennon, KL., Rissler, LJ., & Church, SA. *In press*. Ecogeographic isolation: A reproductive barrier between species and between cytotypes in *Houstonia* (Rubiaceae). Evolutionary Ecology, *in press.*

Google Inc. 2011. Google Earth (version 6.1.0.5001). URL http://www.google.com/earth/index.html.

Grant, V. 1949. Pollination systems as isolating mechanisms in angiosperms. Evolution 3: 82-97.

Grant, V. 1971. *Plant Speciation*. Columbia University Press, New York.

Grundt, HH., Kjølner, S., Borgen, L., Rieseberg, LH., & Brochmann, C. 2006. High biological species diversity in the arctic flora. Proceedings of the National Academy of Sciences 103: 972-975.

Harder, LD., Cruzan, MB., & Thomson, JD. 1993. Unilateral incompatibility and the effects of interspecific pollination for *Erythronium americanum* and *Erythronium albidum* (Liliaceae). Canadian Journal of Botany 71: 353-358.

Hentrich, H., Kaiser, R., & Gottsberger, G. 2010. Floral biology and reproductive isolation by floral scent in three sympatric aroid species in French Guiana. Plant Biology 12: 587-596.

Hoskin, CJ., Higgie, M., McDonald, KR., & Moritz, C. 2005. Reinforcement drives rapid allopatric speciation. Nature 437: 1353-1356.

Husband, BC. 2004. The role of triploid hybrids in the evolutionary dynamics of mixed-ploidy populations. Biological Journal of the Linnean Society 82: 537-546.

Husband, BC. & Schemske, DW. 2000. Ecological mechanisms of reproductive isolation between diploid and tetraploid *Chamerion angustifolium*. Journal of Ecology 88: 689-701.

Husband, BC. & Sabara, HA. 2003. Reproductive isolation between autotetraploids and their diploid progenitors in fireweed, *Chamerion angustifolium* (Onagraceae). New Phytologist 161: 703-713.

Husband, BC., Schemske, DW., Burton, TL., & Goodwillie, C. 2002. Pollen competition as a unilateral reproductive barrier between sympatric diploid and tetraploid *Chamerion angustifolium*. Proceedings of the Royal Society of London B 269: 2565-2571.

Ireland, RR. 1957. Biosystematics of *Erythronium albidum* and *E. mesochoreum*. M.A. dissertation, University of Kansas, Lawrence.

Kaul, RB. 1989. The Status of *Erythronium albidum* and *E. mesochoreum* (Liliaceae) in Nebraska. Transactions of the Nebraska Academy of Sciences 17: 71-79.

Kay, KM. 2006. Reproductive isolation between two closely related hummingbird-pollinated Neotropical gingers. Evolution 60(3): 538-552.

Keller, MJ. & Gerhardt, HC. 2001. Polyploidy alters advertisement call structure in gray treefrogs. Proceedings of the Royal Society of London B 268: 341-345.

Knowlton, N., Weigt, LA., Solórzano, LA., Mills, DK., & Berminghan, E. 1993. Divergence in proteins, mitochondrial DNA, and reproductive compatibility across the Isthmus of Panama. Science 260: 1629-1632.

Levin, DA. 1975. Minority cytotype exclusion in local plant populations. Taxon 24(1): 35-43.

Levin, DA. 1983. Polyploidy and novelty in flowering plants. The American Naturalist 122: 1-25.

Lumaret, R. & Barrientos, E. 1990. Phylogenetic relationships and gene flow between sympatric diploid and tetraploid plants of *Dactylis glomerata* (Gramineae). Plant Systematics and Evolution 169: 81-96.

Marks, GE. 1966. The origin and significance of intraspecific polyploidy: experimental evidence from *Solanum chacoense*. Evolution 20: 552-557.

Mayr, E. 1942. *Systematics and the Origin of Species.* Columbia University Press, New York.

McClain, WE., Ebinger, JE., & Koelling, AC. 1999. Status and distributon of *Erythronium mesochoreum* Knerr (Liliaceae) in Illinois. Castanea 64(4): 346-349.

McCullagh, P. & Nelder, JA. 1989. *Generalized Linear Models.* Chapman & Hall, London.

Michener, CD., & Rettenmeyer, CW. 1956. The ethology of *Andrena erythronii* with comparative data on other species (Hymenoptera, Andrenidae). The University of Kansas Science Bulletin 37: 645-684.

Muller, RN. 1979. Biomass accumulation and reproduction in *Erythronium albidum*. Bulletin of the Torrey Botanical Club 106 (4): 276-283.

Nagy, ES. & Rice, KJ. 1997. Local adaptation in two subspecies of an annual plant: Implications for migration and gene flow. Evolution 51: 1079-1089.

Oksanen, J., Blanchet, FG., Kindt, R., Legendre, P., O'Hara, RB., Simpson, GL., Solymos, P., Stevens, MHH., & Wagner, H. 2011. vegan: Community Ecology Package. R package version 1.17-6. http://CRAN.R-project.org/package=vegan

Oswald, BP. & Nuismer, SL. 2011. A unified model of autopolyploid establishment and evolution. The American Naturalist 178: 687-700.

Otto, SP. 2007. The evolutionary consequences of polyploidy. Cell 131:452-462.

Otto, SP., & Whitton, J. 2000. Polyploid incidence and evolution. Annual Review of Genetics 34: 401-437.

Parisod, C., Holderegger, R., & Brochmann, C. 2010. Evolutionary consequences of autopolyploidy. New Phytologist 186: 5-17.

Pascarella, JB. 2007. Mechanisms of prezygotic reproductive isolation between two sympatric species, *Gelsemium rankinii* and *G. sempervirens* (Gelsemiaceae), in the southeastern United States. American Journal of Botany 94(3): 468-476.

Petit, C., Bretagnolle, F., & Felber, F. 1999. Evolutionary consequences of diploid-polyploid hybrid zones in wild species. Trends in Ecology and Evolution 14(8): 306-311.

Pianka, ER. 1974. Niche overlap and diffuse competition. Proceedings of the National Academy of Sciences 71(5): 2141-2145.

Pires, JC. & Gaeta, RT. 2011. Structural and functional evolution of resynthesized polyploids. pp. 195-214 *in* Schmidt, R. & Bancroft, I. (eds.) *Genetics and Genomics of the Brassicaceae*. Springer, New York.

R Development Core Team. 2010. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Rahmé, J., Widmer, A., & Karrenberg, S. 2009. Pollen competition as an asymmetric reproductive barrier between two closely related *Silene* species. Journal of Evolutionary Biology 22: 1937-1943.

Ramsey, J. 2011. Polyploidy and ecological adaptation in wild yarrow. Proceedings of the National Academy of Sciences 108 (17): 7096-7101.

Ramsey, J. & Schemske, DW. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annual Review of Ecology and Systematics 29: 467-501

Ramsey, J., Bradshaw, HD., & Schemske, DW. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). Evolution 57(7): 1520-1534.

Rieseberg, LH. 1997. Hybrid origins of plant species. Annual Review of Ecology and Systematics 28: 359-389.

Rieseberg, LH. & Willis, JH. 2007. Plant speciation. Science 317 (5840): 910-914.

Rieseberg, LH., Desrochers, AM., & Youn, SJ. 1995. Interspecific pollen competition as a reproductive barrier between sympatric species of *Helianthus* (Asteraceae). American Journal of Botany 82(4): 515-519.

Robertson, KR. 1966. The genus *Erythronium* (Liliaceae) in Kansas. Annals of the Missouri Botanical Garden 53(2): 197-204.

Rundle, HD. & Schluter, D. 1998. Reinforcement of stickleback mate preferences: sympatry breeds contemp. Evolution 52 (1): 200-208.

Schemske, DW., Willson, MF., Melampy, MN., Miller, LJ., Verner, L., Schemske, KM., & Best, LB. 1978. Flowering ecology of some spring woodland herbs. Ecology 59(2): 351-366.

Schliewen, U., Rassmann, K., Markmann, M., Markert, J., Kocher, T. & Tautz, D. 2001. Genetic and ecological divergence of a monophyletic cichlid species pair under fully sympatric conditions in Lake Ejagham, Cameroon. Molecular Ecology 10: 1471-1488.

Segraves, KA. & Thompson, JN. 1999. Plant polyploidy and pollination: Floral traits and insect visits to diploid *Heuchera grossularifolia*. Evolution 53: 1114-1127.

Segraves, KA., Thompson, JN., Soltis, PS. & Soltis, DE. 1999. Multiple origins of polyploidy and the geographic structure of *Heuchera grossulariifolia*. Molecular Ecology 8: 253-262.

Sobel, JM., Chen, GF., Watt, LR., & Schemske, DW. 2010. The biology of speciation. Evolution 64(2): 295-315.

Soltis, DE. & Soltis, PS. 1993. Molecular data and the dynamic nature of polyploidy. Critical Reviews in Plant Sciences 12(3): 243-273.

Soltis, PS. & Soltis, DE. 2009. The role of hybridization in plant speciation. Annual Review of Plant Biology 60: 561-588.

Soltis, DE., Soltis, PS., Schemske, DW., Hancock, JF., Thompson, JN., Husband, BC., & Judd, WS. 2007. Autopolyploidy in angiosperms: have we grossly underestimated the number of species? Taxon 56: 13-30.

Swanson, R., Edlund, AF., & Preuss, D. 2004. Species specificity in pollen-pistil interactions. Annual Review of Genetics 38: 793-818.

Widmer, A., Lexer, C., & Cozzolino, S. 2009. Evolution of reproductive isolation in plants. Heredity 102: 31-38.

Williams, JH., Friedman, WE., & Arnold, ML. 1999. Developmental selection within the angiosperm style: Using gamete DNA to visualize interspecific pollen competition. Proceedings of the National Academy of Sciences 96: 9201-9206.

Wood, TE., Takebayashi, N., Barker, MS., Mayrose, I., Greenspoon, PB., & Rieseberg, LH. 2009. The frequency of polyploid speciation in vascular plants. Proceedings of the National Academy of Sciences 106(33): 13875-13879.

Xiang, Q-Y., Soltis, DE., Soltis, PS., Manchester, SR., & Crawford, DJ. 2000. Timing the eastern Asian-eastern North American floristic disjunction: Molecular clocks confirm paleontological estimates. Molecular Phylogenetics and Evolution 15: 462-472.