doi:10.1111/evo.12666

Hybridization and reproductive isolation between diploid *Erythronium mesochoreum* and its tetraploid congener *E. albidum* (Liliaceae)

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Received May 12, 2014
Accepted April 13, 2015

Polyploidy has played an important role in angiosperm diversification, but how polyploidy contributes to reproductive isolation remains poorly understood. Most work has focused on postzygotic reproductive barriers, and the influence of ploidy differences on prezygotic barriers is understudied. To address these gaps, we quantified hybrid occurrence, interspecific self-compatibility differences, and the contributions of multiple pre- and postzygotic barriers to reproductive isolation between diploid *Erythronium mesochoreum* (Liliaceae) and its tetraploid congener *Erythronium albidum.* Reproductive isolation between the study species was nearly complete, and naturally occurring hybrids were infrequent and largely sterile. Although postzygotic barriers effected substantial reproductive isolation when considered in isolation, the study species' spatial distributions and pollinator assemblages overlapped little, such that interspecific pollen transfer is likely uncommon. We did not find evidence that *E. albidum* and *E. mesochoreum* differed in mating systems, indicating that self-incompatibility release may not have fostered speciation in this system. Ultimately, we demonstrate that *E. albidum* and *E. mesochoreum* are reproductively isolated by multiple, hierarchically-operating barriers, and we add to the currently limited number of studies demonstrating that early acting barriers such as pollinator-mediated isolation can be important for effecting and sustaining reproductive isolation in diploid-polyploid systems.

KEY WORDS: **Flow cytometry, geographic isolation, pollinator-mediated isolation, polyploidy, reproductive barriers, speciation.**

Polyploidy has played an important evolutionary role among angiosperms, influencing mating systems, morphology, and diversification (Stebbins 1950; Grant 1981; Barringer 2007; Wood et al. 2009), and genome duplication is likely the most common mechanism of sympatric speciation (Otto and Whitton 2000). Among plants, traits that cause and maintain reproductive isolation (RI) include prezygotic barriers such as ecogeographic isolation (Nagy and Rice 1997; Ramsey et al. 2003; Glennon et al. 2012) and pollinator-mediated isolation (Grant 1949; Segraves and Thompson 1999; Moe and Weiblen 2012), and postzygotic barriers such as hybrid inviability and sterility (Burton and Husband 2000; Grundt et al. 2006; Borges et al. 2012). Evaluating the relative importance of multiple reproductive barriers is essential to advancing speciation research (Coyne and Orr 2004; Butlin et al. 2012), yet the role of multiple barriers in generating and maintaining RI among closely related plants differing in ploidy (mixed-ploidy systems) remains largely unexplored (Soltis et al. 2003; Husband 2004; Sobel et al. 2010).

The paucity of studies assessing multiple reproductive barriers in mixed-ploidy systems may, in part, be attributed to the long-standing observation that diploid-tetraploid crosses often yield few viable triploid seeds (Marks 1966; Petit et al. 1999),

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making investigations of other barriers seem less important. This phenomenon, termed triploid block, likely results from abnormal triploid endosperm development, and triploid block promotes RI by reducing interploidy gene flow (Ramsey and Schemske 1998). However, the strength of triploid block is taxon-dependent (Ramsey and Schemske 1998). Furthermore, while triploid block can produce near-instantaneous RI between diploids and neotetraploids, it may simultaneously decrease neotetraploids' chances of establishment, via a process called minority cytotype disadvantage (Levin 1975; Coyne and Orr 2004; Husband 2004). Minority cytotype disadvantage occurs when neotetraploids, outnumbered by diploids in mixed-ploidy populations, suffer reduced fitness from frequent intercytotype matings that produce low-fitness triploids (Levin 1975). Assortative mating reduces this inter-cytotype crossing, fostering self-sustaining neotetraploid populations and, potentially, speciation (Coyne and Orr 2004).

Polyploids may overcome minority cytotype disadvantage via a release from self-incompatibility and an accompanying increase in self-fertilization (Husband et al. 2012). While not all polyploids are self-compatible, genome duplication is often associated with increased self-compatibility (Stebbins 1950). Numerous mechanisms, including the breakdown of gametophytic self-incompatibility systems and reductions in inbreeding depression due to the presence of multiple gene copies, may cause self-incompatibility release (Lande and Schemske 1985; Barringer 2007). Indeed, self-fertilization has been shown to be more common among polyploid angiosperms, versus diploids (Barringer 2007; but see Mable 2004). Assessing self-compatibility differences between polyploids and their diploid progenitors can help us infer whether self-incompatibility release, and a subsequent increase in self-fertilization, may have promoted polyploid speciation.

In addition to causing self-incompatibility release, genome duplication can also facilitate prezygotic reproductive isolation, and thus, assortative mating between polyploids and their progenitors (Levin 1983; Levin 2002; Ramsey 2011). Polyploidy is associated with alterations in habitat tolerances, species distributions, and flowering phenology (Bretagnolle and Thompson 1996; Husband and Sabara 2004; Brochmann et al. 2004; Jersáková et al. 2010; Ramsey 2011; Glennon et al. 2012; but see Glennon et al. 2014), which may promote RI by limiting intercytotype pollination (Levin 2002). Despite the potential evolutionary relevance of prezygotic barriers, investigations of multiple pre- and postzygotic barriers in mixed-ploidy systems remain scarce (Husband and Sabara 2004; Sobel et al. 2010).

Diploid *Erythronium mesochoreum* Knerr (Liliaceae) ($2n = 2x = 22$) and its tetraploid congener *E. albidum* Nutt. ($2n = 4x = 44$) are North American perennial forbs that produce a single, insect-pollinated flower in early spring, set a single, multiseeded fruit, and senesce by early summer. This species pair is well suited for investigation of mechanisms of RI, since for decades it was unclear whether these taxa were distinct species, as several of their morphological traits can overlap (Kaul 1989). They are now recognized as separate species, but neither the frequency of hybridization nor the reproductive barriers between these taxa have been thoroughly examined. Previous research has indicated that *E. albidum* flowers later than *E. mesochoreum* and that these species may have different pollinators (Michener and Rettenmeyer 1956; Ireland 1957; Banks 1980). This species pair therefore offers an opportunity to evaluate the relative importance of pre- versus postzygotic barriers for RI. The goals of our study were to address the paucity of assessments of multiple reproductive barriers in mixed-ploidy systems by quantifying RI between these taxa, based on both triploid hybrid occurrence and the strength of multiple pre- and postzygotic barriers, and by assessing potential interspecific self-incompatibility differences. We first used flow cytometry to record the frequency of hybrids across multiple contact zones. Next, we quantified the strength of: (1) geographic isolation; (2) flowering asynchrony; (3) pollinator-mediated isolation; (4) heterospecific crossing barriers (5) F1 seed fitness (using seed mass as a proxy); and (6) triploid hybrid sterility. Finally, we compared seed production from selfed hand-pollinations of both species to assess self-compatibility differences.

Materials and Methods

STUDY SPECIES

Erythronium albidum inhabits woodlands from the eastern and central United States north to Ontario, Canada, whereas *E. mesochoreum* is primarily restricted to tallgrass prairies in the central United States (Allen and Robertson 2002) (Fig. S1). Populations of these species can abut at prairie-forest ecotones and can intergrade widely in intermediate habitats (e.g., oak savannah; Kaul 1989; McClain et al. 1999). Both species are perennial spring wildflowers, producing one perfect flower per plant that remains open for ~1 week (K.R., pers. observation). The pollen:ovule ratio of *E. albidum* (966:1) indicates that it is self-compatible and also adapted for outcrossing (Cruden 1977; Banks 1980). Very little is known about the reproductive biology of *E. mesochoreum* (Kaul 1989).

Several lines of evidence suggest that diploid *E mesochoreum* likely gave rise to tetraploid *E. albidum*. Among all *Erythronium* species, only *E. albidum*, *E. mesochoreum*, and *E. propullans* ($2n = 4x = 44$) have the base chromosome number $x = 11$ (Allen et al. 2003). However, *E. propullans* is endemic to Minnesota and is likely a recent derivative of *E. albidum* (Pleasants and Wendel 1989). ITS sequences indicate that *E. albidum* and *E. mesochoreum* are closely related and are possibly sister taxa (Allen et al. 2003). Taken together, these data suggest that the $x = 11$ base chromosome number originated only once

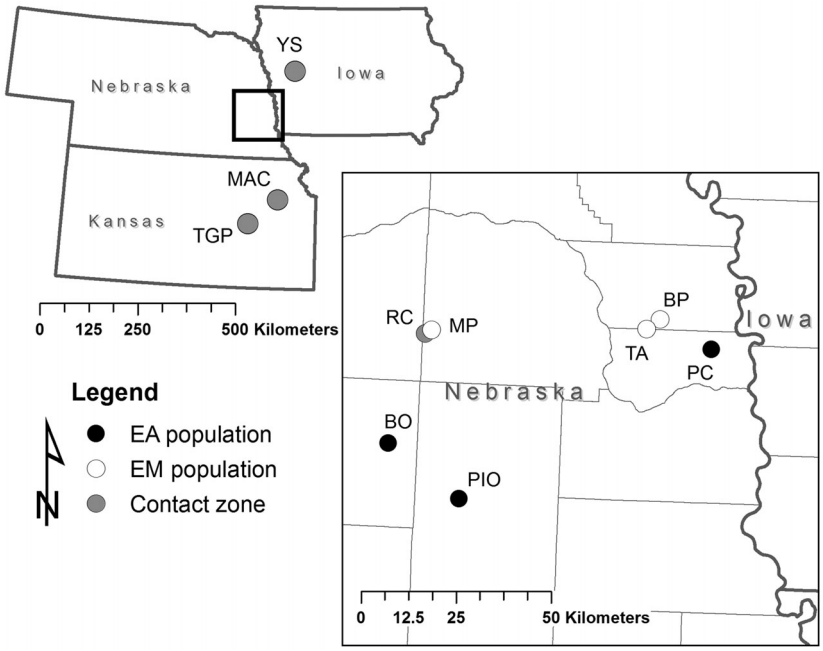

Figure 1. Field sites used for studies of hybridization and reproductive isolation between *Erythronium albidum* (EA) and *Erythronium mesochoreum* (EM). EA sites—Bur Oak Wildlife Management Area (BO; 40.896°N, 97.000°W), Papio Creek (PC; 41.149°N, 96.002°W), and Pioneers Park (PIO; 40.772°N, 96.772°W). Contact zones—Yellow Smoke Park (YS; 42.042°N, 95.323°W), MacLennan Park (MAC; 39.067°N, 95.733°W), Red Cedar Recreation Area (RC; 41.169°N, 96.880°W), and Tallgrass Prairie National Preserve (TGP; 38.492°N, 96.589°W). TGP was not sampled systematically for hybrid presence because of the distance between *E. albidum* and *E. mesochoreum* populations. EM sites—Bauermeister Prairie (BP; 41.215°N, 96.166°W), Madigan Prairie (MP; 41.169°N, 96.881°W), and Te Amo Prairie (TA; 41.192°N, 96.208°W).

(Allen et al. 2003). We hypothesize that *E. albidum* arose from *E. mesochoreum* via autopolyploid speciation, as the two species share morphological and chromosomal similarities. Aside from *E. propullans*, *E. albidum* and *E. mesochoreum* are the only eastern North American *Erythronium* species bearing white flowers. Furthermore, *E. albidum*'s chromosomal morphology mirrors that of *E. mesochoreum*. *Erythronium mesochoreum* has eight meta- or submetacentric chromosomes and 14 acrocentric chromosomes, while 16 of *E. albidum*'s chromosomes are meta- or submetacentric and 28 are acrocentric (Robertson 1966). Alternatively, *E. albidum* may be an allopolyploid, with *E. mesochoreum* as one progenitor species. *Erythronium albidum*'s chromosomes form bivalents during meiosis (Cooper 1939), a characteristic more typical of allo- versus autopolyploids (Coyne and Orr 2004). *Erythronium albidum* also inhabits woodlands and has mottled leaves, characteristics shared by other eastern North American *Erythronium* species, but uncommon for *E. mesochoreum*. Finally, it is possible that *E. mesochoreum* is a polyhaploid derivative of *E. albidum*. However, this is unlikely, since polyhaploids typically exhibit low fitness and cytological instability (Stebbins 1980; Ramsey and Schemske 2002). Triploid hybrids of *E. albidum* and *E. mesochoreum*, identified via karyotypes and genome size measurements, were collected in eastern Nebraska in 1996 (R.B. Kaul, unpublished data; NEB 306411). We identified *Erythronium* populations at 10 study sites in Nebraska, Kansas, and Iowa (Fig. 1). *Erythronium albidum* populations occurred at Bur Oak Wildlife Management Area (BO), Papio Creek (PC), and Pioneer's Park (PIO), while *E. mesochoreum* occurred at Bauermeister Prairie (BP), Madigan Prairie (MP), and Te Amo Prairie (TA). MacLennan Park (MAC), Red Cedar Recreation Area (RC), Tallgrass Prairie National Preserve (TGP), and Yellow Smoke Park (YS) were obvious contact zones, as the two species were often located within two meters of one another. Herein, populations are defined in a broad sense, as aggregations of co-occurring individuals.

HYBRID FREQUENCY

In 2010 and 2011, we used targeted and systematic sampling of *Erythronium* leaves to assess hybrid occurrence at the contact zones (Fig. 1). The study species were the only *Erythronium* species present at these sites. *Erythronium albidum* leaves are generally mottled and mostly flat, whereas *E. mesochoreum* typically has folded, nonmottled leaves (Allen and Robertson 2002). We conducted targeted leaf sampling by searching for and collecting leaves with intermediate morphology (putative hybrids) at each contact zone ($n_{\text{total}} = 128$). We also systematically collected leaves at MAC, RC, and YS ($n_{\text{total}} = 224$; Fig. 1) by establishing multiple transects spanning the contact zone at each site and collecting the nearest *Erythronium* leaf every two meters along each transect.

Flow cytometry was used to identify hybrids based on genome size differences. We followed procedure 1A from Doležel et al. (2007), using *Allium cepa* cv. "Ailsa Craig" as the internal reference standard (Bennett et al. 2000), Galbraith's nuclear isolation buffer (Galbraith et al. 1983), propidium iodide (50 μL, 1.0 mg/mL), and RNase (75 μL, 0.5 mg/mL). We analyzed the samples with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and calculated relative fluorescence (RF) by dividing the mean fluorescence intensity of the sample peak by that of the standard peak. RF values of the leaves from contact zones were compared to those of 54 *E. albidum* and 56 *E. mesochoreum* reference leaves collected at sites containing only one study species. Contact zone leaves were scored as *E. albidum* or *E. mesochoreum* if they fell within the range of the reference leaves' RF values. We anticipated that hybrids' RF would be intermediate to the parental species, and we created a predicted range of hybrid RF values by using the average of the reference leaves' lowest *E. albidum* RF value and lowest *E. mesochoreum* RF value as the lower bound for hybrid RF, and the average of the highest *E. albidum* and *E. mesochoreum* RF values as the upper bound (modified from Husband and Schemske 1998). Leaves with RF values within this range were scored as hybrids.

The most direct way to quantify total RI is to compare the observed hybrid frequency at contact zones to the expected frequency under random mating, estimated as two times the product of the diploid and tetraploid frequencies (Husband and Sabara 2004; Sobel and Chen 2014, eq. S1). We used our systematic leaf sampling data to calculate total RI in this manner, and we compared this value of total RI to the value obtained from our assessments of reproductive barriers (described in *Reproductive Barriers*). Because we did not collect a priori data on the frequency of either species at contact zones, we estimated their frequencies as 0.5 and calculated mean total RI across all contact zones.

REPRODUCTIVE BARRIERS

We quantified the contributions of six reproductive barriers between the study taxa using Sobel and Chen's (2014) methodology (see Supporting Information for detailed explanations of RI calculations). We first calculated a RI value for each barrier, which represents that barrier's proportional reduction in gene flow, relative to expectations under random mating. Next, we calculated total RI for each study species using Sobel and Chen's (2014) equation (4E). Finally, because reproductive barriers act sequentially, we calculated the absolute contribution of each barrier to total RI, which takes into account the effects of all previously acting barriers.

Geographic isolation

Because geographic isolation in this system depends on the ability of pollinators to make interspecific flights, we considered populations of the study species to be geographically isolated if the distance between heterospecific populations was greater than the foraging ranges of the study species' pollinator assemblages. We calculated a single predicted foraging range for the pollinator assemblage of each plant species, using our floral visitor data (see *Pollinator-Mediated Isolation*). Because bees were the dominant visitors of both species, only bees were included in our analyses. Foraging range was estimated for each bee species using measurements of the distance between the wing bases (intertegular span). Intertegular (IT) span is directly related to thoracic flight musculature, making it suitable for estimating foraging ranges (Cane 1987; Greenleaf et al. 2007). Average IT span for each bee species was obtained from measurements of ≥ 5 females (depending on specimen availability). Mean IT span for the entire bee assemblage was calculated by weighting by the bee species' relative abundance, aggregated across all sites, for each plant species separately. Finally, we used the mean IT spans to calculate a single foraging range estimate for the bee assemblage of each *Erythronium* species, using Greenleaf et al.'s (2007) maximum homing distance equation.

We mapped Nebraska populations of both *Erythronium* species in ArcMap 10.2 (ESRI, 2013), using records from the University of Nebraska's herbarium and the authors' field surveys. A focal population was considered reproductively isolated from a heterospecific population if the distance between the populations exceeded the aggregate foraging range of the focal species' bee assemblage. We used equation (4C) from Sobel and Chen (2014) to quantify $RI_{geographic}$ for each plant species (eq. S2).

Flowering asynchrony

We quantified flowering asynchrony at two scales in order to evaluate both larger scale spatial and temporal trends as well as patterns at the finer-scales at which ecological interactions occur. The larger scale assessment used herbarium records to evaluate interspecific differences in flowering phenology after controlling for geographic differences in temperature and precipitation. We used *E. albidum* ($n = 124$) and *E. mesochoreum* ($n = 130$) herbarium accessions collected in Nebraska, Iowa, Kansas, and Missouri from 1873–2007 (Table S1). We recorded the locality and date for each accession containing ≥ 1 flowering plant and georeferenced the accessions with Google Earth (Google Inc., 2011). For accessions lacking detailed localities, coordinates of the county seat for the county of collection were used. We assigned each plant an ordinal flowering day (OFD) by converting the collection date to the ordinal day.

We assessed larger scale flowering asynchrony by testing for interspecific differences in OFD. Because the herbarium accessions were collected over a large geographic area, we controlled for climatic differences by assigning each accession a single mean annual temperature (MAT) and mean annual precipitation (MAP)

value, based on the collection locality and data from the High Plains Regional Climate Center (www.hprcc.unl.edu). For each accession, we located the nearest weather station where ≥ 90% of the daily average temperature and daily total precipitation values were available for ≥ 70 years. To calculate a single MAT and MAP value for each accession, yearly MAT and MAP values were averaged across the entire period of record (through 2011). The mean time periods over which MAT and MAP were calculated were 89 and 88 years, respectively, to represent the long-term, regional climate. Although we also could have used the latitude and longitude of each accession to account for climatic differences, MAT and MAP are highly correlated with latitude and longitude, and models with MAT and MAP had nearly identical Akaike information criterion (AIC) scores (data not shown). All analyses in this study were carried out in R (R Development Core Team 2010). A general linear model (with Type III tests of fixed effects) was used to assess the effects of species, collection year, MAT, and MAP on OFD. The full model included all main effects and interactions. Beginning with the highest order interaction term, we removed nonsignificant terms one at a time based on *F*-tests (Crawley 2007). Because the herbarium records represented broad phenological trends for *E. albidum* and *E. mesochoreum*, we did not include them in our RI calculations.

To assess fine-scale flowering asynchrony, we tracked flowering phenology across a total of 20 quadrats (∼ 1 m^2) across seven sites in 2010 and 2011 (1–3 quadrats/site)—BP, BO, MP, PC, PIO, TA, and RC (Fig. 1). Each quadrat contained plants of one study species (4–53 flowering plants/quadrat). We surveyed each quadrat ≥1 time weekly, tagging emerging plants and scoring each plant as "flowering" or "not flowering," until all plants senesced. Because few *E. albidum* flowered in 2011 at RC, we tracked phenology across a ∼0.5 ha area and treated this as one quadrat ($n = 36$ plants).

We calculated the proportion of flowering plants in each quadrat during each calendar week and used Pianka's overlap index:

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

(Pianka 1974) to quantify mean interspecific flowering overlap (O_{jk}) for every pairwise *E. albidum*—*E. mesochoreum* quadrat comparison in both study years.

We assessed the significance of interspecific flowering asynchrony using null models modified from Ashton et al. (1988). We compared the observed O_{jk} values with a null distribution of 999 overlap values generated by randomly shifting the position of peak flowering for each quadrat within the observed flowering season, while preserving the shape of each distribution. Significant asynchrony was determined if the observed O_{jk} fell at or below the fifth percentile of the null distribution. We quantified $RI_{asynchrony}$ in each year from the field-collected data using equation (4S1) from Sobel and Chen (2014) and calculated the mean value (eq. S3).

Pollinator-mediated isolation

Insects visiting the study species were captured at BO (*E. albidum* only), MP (*E. mesochoreum* only), and RC (both species) over four collecting trips in 2010 and five trips in 2011 (∼15 person-hours in total), with approximately equal effort for *E. albidum* and *E. mesochoreum*. During each trip, we walked throughout the site at hours of peak pollinator activity and opportunistically captured insects contacting the reproductive structures of *Erythronium* plants for 1–2 person-hours. Insects were identified by the USDA-ARS Systematic Entomology Laboratory. Because we did not verify that these insects were carrying pollen, we refer to them as floral visitors.

We calculated a single O_{jk} value (eq. (1)) between the floral visitor assemblages of the study species using data aggregated across all sites and years. We assessed the significance of interspecific differences in the study plants' insect assemblages using a null model developed under the hypothesis that all insects were equally available to both species. Working in R's "vegan" package (Oksanen et al. 2011), we randomly assigned each insect to a plant species. We kept constant both the total number of insects per plant species and insect abundances to accurately reflect insect species composition and visitation patterns. We considered there to be significant interspecific differences in the floral visitor assemblages if the calculated O_{jk} value fell at or below the fifth percentile of the distribution of 999 randomized values. Using Sobel and Chen's (2014) equation (4C), we quantified $RI_{pollinator}$ for the entire floral visitor assemblage, aggregated over both years (eq. S2).

Our analyses assume no site-specific differences in the pollinator species pools. This likely holds true for MP and RC, which are located approximately 350 m apart, but BO is more isolated. In addition, both MP and BO contained only one *Erythronium* species. To further explore these limitations, we reassessed the significance of our data using the null model outlined above, with the dataset restricted to insects captured at both an *E. albidum* and an *E. mesochoreum* site.

Heterospecific crossing barriers

To test for reductions in hybrid seed set (number of seeds, divided by the total number of ovules per fruit), potentially due to triploid block, we performed conspecific ($n = 95$), and heterospecific ($n = 113$) hand-pollinations of the study species at BO and MP in 2010 and 2011. We emasculated budding plants to prevent selfing, then applied either heterospecific or conspecific pollen to each plant after floral anthesis. Donor pollen was collected immediately prior

to each hand-pollination. Because we had not previously determined the timing of stigma receptivity, we hand-pollinated each plant twice: 48–72 hours after emasculation, and 48–72 hours after the first application. Stigmas were thoroughly coated with pollen, and pollinators were excluded with mesh exclosures. We used 30 emasculated, nonpollinated plants as a negative control, of which one *E. albidum* plant set seed. Previous studies have not recorded apomictic seed production for *E. albidum* (Schemske et al. 1978; Banks 1980), and we believe that the plant's exclosure may have failed. We verified the efficacy of our hand-pollination technique by comparing seed set from conspecific hand-pollinations to that of naturally occurring fruits (Supporting Information).

Seed set was analyzed with generalized linear models (McCullagh and Nelder 1989) as a binomial process, $m_j \sim \text{Binomial}(N_j, p_j)$, where m_j represents the number of seeds set per fruit, N_j represents the number of ovules per fruit, and p_j represents the probability of an ovule setting seed. Because the residual deviance values were larger than the residual degrees of freedom, we fit the overdispersion parameter (Crawley 2007). Seed set was modeled as a function of the fixed effects of cross type (conspecific vs heterospecific), maternal species (*E. albidum* vs *E. mesochoreum*), and their interaction separately for each year (2010 and 2011). A priori linear contrasts were used to investigate differences in seed set among specific treatment groups. We quantified $RI_{crossing}$ using Sobel and Chen's (2014) equation (4A) and calculated the mean value (eq. S4).

F1 seed mass

Because reduced hybrid seed mass could indicate developmental abnormalities in the embryo or endosperm (Howard 1939; Haig and Westoby 1991), potentially due to triploid block, we compared mean seed mass per fruit between fruits resulting from the conspecific and heterospecific hand-pollinations. A general linear model was used to model the effects of maternal species, cross-type, and their interaction separately for 2010 and 2011. Seed mass was log-transformed to improve normality. We quantified $RI_{seed\ mass}$ (eq. 4A, Sobel and Chen 2014) by comparing mean seed masses from conspecific and heterospecific hand-pollinations of *E. albidum* and *E. mesochoreum*, separately (eq. S4). RI values were averaged across both years.

Hybrid sterility

In 2012, we compared pollen viability from 10 *E. mesochoreum* plants to that of three naturally occurring hybrids, identified via flow cytometry. We were unable to collect pollen from *E. albidum*, so we obtained *E. albidum* pollen viability values from Banks (1980). Two undehisced anthers were collected from each plant and allowed to dehisce in open microcentrifuge tubes under ambient laboratory conditions. Using a method similar to that of Banks (1980), we considered pollen grains viable if they stained dark blue after the addition of lactophenol blue solution (Kearns and Inouye 1993). We evaluated the significance of interspecific differences in pollen viability by examining whether the 95% confidence intervals calculated for hybrids and the parental species overlapped. A calculated a single, mean $RI_{pollen\ viability}$ value was calculated using Sobel and Chen's (2014) equation (4A) (eq. S4).

ASSESSING SELF-COMPATIBILITY

In 2010 and 2011, we compared seed set between self-pollinated *E. albidum* ($n_{2010} = 4$; $n_{2011} = 14$) and *E. mesochoreum* ($n_{2010} = 5$; $n_{2011} = 14$) individuals at BO and MP, using the hand-pollination procedure outlined above. Immature anthers from each study plant were collected, and the pollen was allowed to dehisce in the laboratory. We verified that this method resulted in viable pollen using lactophenol blue (data not shown). We used a generalized linear model with a quasibinomial error distribution to compare selfed seed set between *E. albidum* and *E. mesochoreum* separately for each year.

Results

HYBRID FREQUENCY

Mean (± 1 standard error (SE)) RF of the reference leaves was 4.53 ± 0.01 (range: 4.22–4.70) for *E. albidum* and 3.02 ± 0.02 (range: 2.62–3.25) for *E. mesochoreum*. We established the expected range of hybrid RF values as 3.42–3.98. In total, 334 leaves from contact zones were assessed with flow cytometry. Of these, 39 had RF values > 4.70 (the highest RF from the reference leaves; Fig. S2). These leaves had a mean (± 1 SE) RF of 4.78 ± 0.01 and were scored as *E. albidum*. One leaf had a RF value < 2.62 (the lowest *E. mesochoreum* reference leaf's RF) and was scored as *E. mesochoreum* (Fig. S2).

Eight individuals collected at RC via targeted sampling were scored as hybrids (Figs. S2 and S3), as their RF values fell within the predicted range of hybrid values. Mean hybrid RF (± 1 SE) was 3.91 ± 0.01 (range: 3.85–3.97). The systematic sampling revealed one individual at RC with a RF slightly higher than the highest predicted hybrid RF value (Fig. S2). This individual (RF = 4.04) was scored as hybrid (Fig. 2). Our evaluation of total RI based on hybrid frequency was 1 for both YS and MAC, and 0.982 for RC (mean $RI_{total} = 0.994$).

REPRODUCTIVE BARRIERS

Geographic isolation

We identified 16 *E. albidum* populations and 10 *E. mesochoreum* populations in Nebraska (Table S2). The aggregate foraging ranges were 636 m for the *E. albidum* bee assemblage and 605 m for the *E. mesochoreum* bee assemblage. Two *E. albidum*

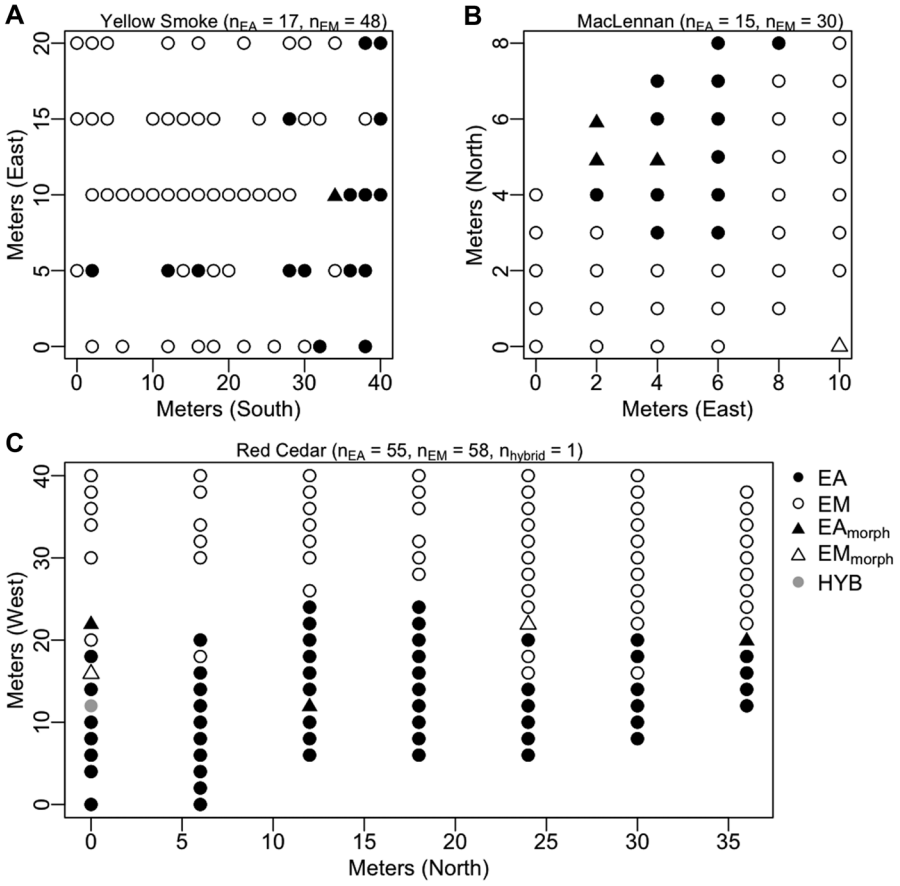

Figure 2. Schematic maps of the locations along transects of *E. albidum* (EA), *E. mesochoreum* (EM), and hybrid (HYB) plants. Plants were identified using flow cytometry (EA, EM) or, if leaf tissue was degraded, photographs, and descriptions of leaf morphology (EA_{morph}, EM_{morph}). Leaves were collected along parallel transects 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). Gaps represent points along the transects that had no *Erythronium* plants nearby.

populations were located within 636 m of a heterospecific population ($RI_{geographic} = 0.875$), while three *E. mesochoreum* populations were located within 605 m of a heterospecific population ($RI_{geographic} = 0.700$) (Table 1, Fig. 5A).

Flowering asynchrony

For *E. albidum* herbarium accessions, flowering ranged from March 24–May 27. Flowering for *E. mesochoreum* ranged from March 3–May 2. The final model consisted of the main effects of species, collection year, and mean annual temperature (MAT), which all significantly affected ordinal flowering day (OFD) (Table 2). After accounting for collection year and MAT, *E. mesochoreum* (mean OFD = 97.7, 95% CI (95.6, 99.8)) flowered 7.11 $\pm$ 1.32 days earlier (model estimate $\pm$ 1 SE) than *E. albidum* (mean OFD = 107.8, 95% CI (105.9, 109.8)). Both species' OFD decreased as collection year increased, flowering on average 0.47 $\pm$ 0.20 days earlier for every 10-year increase in collection year. Both species responded to MAT similarly, with a 2.14 $\pm$ 0.29 day decrease in OFD per degree increase in MAT.

In 2010, the mean peak of flowering for the field-surveyed *E. albidum* quadrats occurred on ordinal day 98.0 (95% CI: 85.3, 110.7), 0.70 days after the mean peak flowering of *E. mesochoreum* ($\bar{x} = 97.3$, 95% CI: 94.24, 100.26). The initiation of flowering was not recorded in 2010. In 2011, mean flowering initiation occurred on ordinal day 100.6 (95% CI: 97.6, 103.5) for *E. albidum*, 4.4 days after that of *E. mesochoreum* ($\bar{x} = 96.2$, 95% CI: 94.4, 98.1). Mean peak flowering for *E. albidum* quadrats occurred on ordinal day 106.4 (95% CI: 99.9, 112.9), 5.8 days after that of *E. mesochoreum* ($\bar{x} = 100.6$, 95% CI: 99.5, 101.7). Mean ($\pm$ 1 SE) O_{jk} was 0.861 $\pm$ 0.048 in 2010 and 0.605 $\pm$ 0.045 in 2011 (Fig. 3), with no significant flowering asynchrony between *E. mesochoreum* and *E. albidum* quadrats ($P_{2010} = 0.649$; $P_{2011} = 0.559$). Mean $RI_{asynchrony}$ was 0.174 for *E. albidum* and 0.228 for *E. mesochoreum* (Table 1, Fig. 5).

Table 1. Quantification of the contributions of multiple barriers to reproductive isolation (RI) between *Erythronium albidum* and *Erythronium mesochoreum*.

	Measured RI value		Absolute contribution (AC) to RI		AC to RI in sympatry	
	E. albidum	*E. mesochoreum*	*E. albidum*	*E. mesochoreum*	*E. albidum*	*E. mesochoreum*
Geographic isolation[1]	0.875	0.700	0.875	0.700	–	–
Flowering asynchrony[2]	0.174	0.228	0.022	0.068	0.174	0.228
Pollinator-mediated isolation[1]	0.410	0.767	0.042	0.178	0.339	0.593
Heterospecific crossing barriers[3]	0.285	0.236	0.027	0.020	0.183	0.065
Hybrid seed mass[3]	–0.049	–0.007	–0.003	–0.0005	–0.026	–0.002
Hybrid pollen viability[3]	0.810	0.890	0.034	0.032	0.290	0.110
Total RI			**0.996**	**0.998**	**0.959**	**0.993**

Values were calculated from Sobel and Chen (2014) both with and without the contribution of geographic isolation. Superscripts indicate the equation used to calculate each measured RI value: 1 = equation (4C) (Sobel and Chen 2014), reprinted as equation (S2) (Supporting Information); 2 = equation (4S1) (Sobel and Chen 2014), reprinted as equation (S3) (Supporting Information); 3 = equation (4A) (Sobel and Chen 2014), reprinted as equation (S4) (Supporting Information). Total RI was calculated using the online supplement provided by Sobel and Chen (2014), based on their equation (4E). AC values were computed by first calculating total RI for the focal barrier and all preceding barriers, then subtracting from that value the total RI, including all preceding barriers, but excluding the focal barrier (Sobel and Chen 2014).

Table 2. Analysis of variance table from a general linear model investigating the effects of mean annual temperature, species, and collection year on ordinal flowering day for *E. albidum* and *E. mesochoreum* herbarium accessions collected in Nebraska, Iowa, Missouri, and Kansas.

	D.f.	S.S.	M.S.	F	*P*
Mean annual temperature	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	27475.40	101.90		

Pollinator-mediated isolation

Sixty-nine insects representing 14 species were captured (Table 3). *Andrena carlini* (Andrenidae) was the most frequent *E. albidum* visitor, and *Andrena erythronii* was the most frequent *E. mesochoreum* visitor. Previous workers recorded these two species collecting pollen from *E. albidum* (*Andrena carlini*) and *E. mesochoreum* (*Andrena erythronii*) (Robertson 1929; Michener and Rettenmeyer 1956; Krombein et al. 1979; Banks 1980; Table 3). Three species—*Andrena carlini*, *Osmia pumila* (Megachilidae), and *Ceratina calcarata* (Apidae)—were captured on both study species. Of these insects, *A. carlini* was the most frequent visitor (35% of all recorded visits) but was captured nearly four times more often on *E. albidum* than *E. mesochoreum*. Interspecific overlap (O_{jk}) was 0.23, falling below the lowest value in the null distribution (range: 0.59–0.98), and indicating significant interspecific differences in the insect assemblages ($P < 0.001$). $RI_{pollinator}$ was 0.410 for *E. albidum* and 0.767 for *E. mesochoreum* (Table 1, Fig. 5). After restricting our dataset to consider only the four insect species that were captured at both an *E. albidum* and an *E. mesochoreum* site (*Andrena carlini*, *Bombylius major*, *Ceratina calcarata*, and *Osmia pumila*), O_{jk} rose to 0.49. The restricted model's null distribution of O_{jk} values was above this value (0.50–0.98), confirming the results from the full dataset.

Heterospecific crossing barriers

In both years, mean seed set from conspecific hand-pollinations exceeded that of heterospecific hand-pollinations (Table S3, Fig. 4A, B). Seed set was reduced by 46% in 2010 and 43% in 2011 when *E. albidum* was pollinated with heterospecific, versus conspecific, pollen. Seed set was reduced by 29% in 2010 and 47% in 2011, when *E. mesochoreum* was pollinated with heterospecific, versus conspecific, pollen. $RI_{crossing} = 0.285$ for *E. albidum* and 0.236 for *E. mesochoreum* (Table 1, Fig. 5). Regardless of pollen donor, *E. mesochoreum* had significantly higher mean seed set than *E. albidum* in both years (Table S3, Fig. 4A, B). A significant cross type × maternal species interaction occurred in 2011 only (Table S3).

F1 seed mass

There were no significant differences in average seed mass between fruits resulting from conspecific versus heterospecific crosses in either year (Table S4, Fig. 4C, D). Mean $RI_{seed\ mass}$ was –0.049 for *E. albidum* and –0.007 for *E. mesochoreum* (Table 1, Fig. 5). In 2011, *E. albidum* fruits had significantly greater mean seed mass than *E. mesochoreum* fruits, regardless of pollen donor

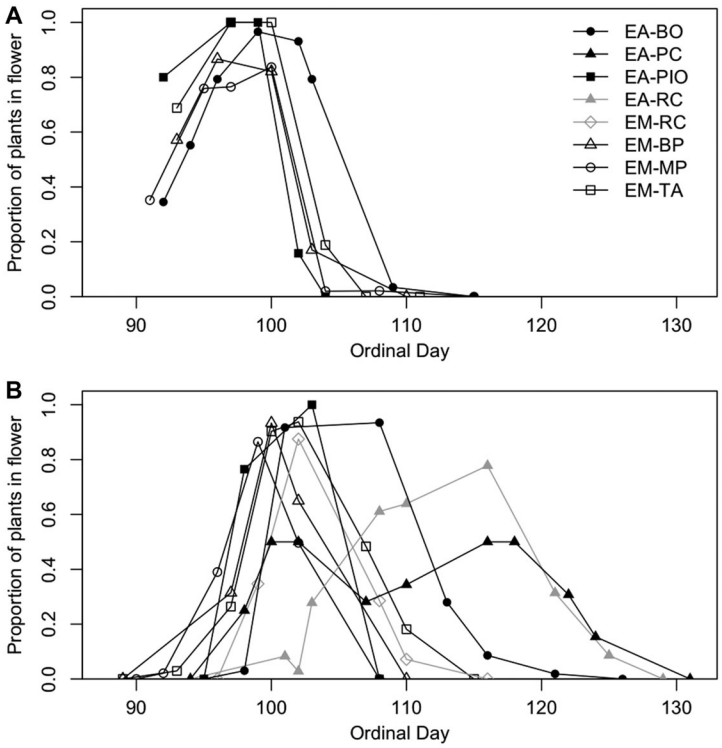

Figure 3. **Flowering phenology overlap for populations of *E. albidum* (EA) and *E. mesochoreum* (EM) at seven eastern Nebraska study sites. Bur Oak Wildlife Management Area (BO), Pioneers Park (PIO), Bauermeister Prairie (BP), Madigan Prairie (MP), and Te Amo Prairie (TA) were surveyed in both 2010 and 2011. Red Cedar Recreation Area (RC) and Papio Creek (PC) were surveyed in 2011 only. Each line represents the flowering progression of a population at one site in either (A) 2010, or (B) 2011. Flowering progression lines for each population were often averaged over several study quadrats. Gray lines indicate data recorded at a zone of species contact. In 2010, we failed to establish plots before the onset of flowering.**

identity ($P < 0.001$, Table S4; Fig. 4D). The cross type × maternal species interaction was nonsignificant in both years (Table S4).

Hybrid sterility

Mean (95% CI) pollen viability for the three hybrids was 6% (0%, 24.6%), significantly lower than the 99% pollen viability (98.4%, 99.2%) of *E. mesochoreum* ($P < 0.001$). Using similar methods, Banks (1980) recorded mean pollen viability of *E. albidum* as 57% (55.7%, 58.9%), which, based on a 95% confidence interval assuming a normal error distribution, is significantly higher than hybrid pollen viability, but significantly lower than *E. mesochoreum* pollen viability. $RI_{pollen\ viability}$ was 0.810 for *E. albidum* and 0.890 for *E. mesochoreum* (Table 1, Fig. 5).

Total reproductive isolation

Total reproductive isolation was 0.996 for *E. albidum* and 0.998 for *E. mesochoreum* when calculated across all measured barriers, and geographic isolation had the greatest absolute contribution to total reproductive isolation (Table 1, Fig. 5A). When calculated taking into account only the barriers that would act in sympatry (i.e., ignoring geographic isolation), RI_{total} was 0.959 for *E. albidum* and 0.993 for *E. mesochoreum*, and pollinator-mediated isolation was the strongest barrier (Table 1, Fig. 5B).

ASSESSING SELF-COMPATIBILITY

In 2010, mean (± 1 SE) seed set was 0.133 ± 0.017 for selfed *E. albidum* fruits and 0.185 ± 0.133 for selfed *E. mesochoreum* fruits. In 2011, mean seed set rose to 0.298 ± 0.034 for *E. albidum* but fell to 0.066 ± 0.033 for *E. mesochoreum*. Selfed *E. albidum* seed set was significantly higher than that of *E. mesochoreum* in 2011 only ($P_{2010} = 0.288$, $P_{2011} = 0.0006$) (Fig. S4).

Discussion

Our detailed assessment of multiple reproductive barriers indicates that *E. albidum* and *E. mesochoreum* are nearly completely reproductively isolated, even when geographic isolation is not considered, and this analysis is borne out by the few triploid hybrids that were discovered. As is the case for many mixed-ploidy taxa, late-acting reproductive barriers were strong, when considered in isolation from other barriers. Hybrid sterility reduced the probability of interspecific gene flow by ~90% for both species,

Table 3. Identity, abundance, and location of insects captured while visiting flowers of *E. albidum* and *E. mesochoreum*.

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

Insect collections were made in 2010 and 2011 at Bur Oak Wildlife Management Area (BO; Seward Co., NE), Madigan Prairie (MP; Saunders Co., NE), and Red Cedar Recreation Area (RC; Saunders Co., NE). Insect identification was provided by the USDA-ARS Systematic Entomology Laboratory. The † symbol indicates that this species was observed collecting pollen on the study plant species by one or more of the following workers: R = Robertson (1929); M = Michener and Rettenmeyer (1956); K = Krombein et al. (1979); B = Banks (1980) (see Literature Cited).

and physiological crossing barriers reduced both species' probabilities of heterospecific gene flow by nearly one-third, compared to expectations under random mating. However, the opportunity for interspecific gene flow to be reduced by these barriers is likely strongly limited by geographic- and pollinator-mediated isolation. Because interspecific RI is nearly complete, we could not determine whether the isolating barriers we observed played a role in polyploid speciation or instead arose during subsequent divergence. There was also no conclusive evidence that *E. albidum* had greater self-compatibility than *E. mesochoreum*, indicating that self-incompatibility release may not have contributed to divergence between these species. Ultimately, we demonstrate that multiple, hierarchically operating barriers play an important role in maintaining isolation between this diploid-tetraploid pair, which underscores the importance that prezygotic barriers play in reducing gene flow in mixed-ploidy systems.

FREQUENCY OF HYBRID OCCURRENCE

We discovered nine hybrids, all of which came from RC. Our estimate of ∼99% RI based on hybrid frequency is consistent with our estimate of ∼99% total RI for both species, calculated based on the strength of multiple reproductive barriers. These data indicate that very little interspecific gene flow likely occurs.

Erythronium albidum's genome size did not increase in direct proportion to ploidy. Despite having twice the chromosome complement, *E. albidum*'s mean RF (proportional to DNA content) was only ∼1.5 times that of *E. mesochoreum*. This phenomenon, termed genome downsizing, is widespread across angiosperms (Leitch and Bennett 2004). Genome modifications can occur rapidly after genome duplication, though the selection pressures underlying this phenomenon remain poorly understood (Soltis et al. 2003; Leitch and Leitch 2008). We are confident that our cytometry-based assessment of hybrids was accurate, as the distribution of RF values from contact zone leaves exhibited peaks corresponding closely to our predicted ranges for *E. mesochoreum* and hybrids. The individuals falling outside of the predicted ranges for the parental species and hybrids may be aneuploid, or may have experienced introgression, which can affect genome size (Baack and Rieseberg 2007). However, hybrid sterility in this system likely strongly reduces rates of backcrossing. Interestingly, many leaves scored as *E. albidum* had RF values higher than the upper bound of our reference leaves' values. *Erythronium albidum* also exhibited more interpopulation variation in RF (and, thus, genome size), suggesting that rates of aneuploid formation and gene flow between *E. albidum* and *E. mesochoreum* may be locally variable.

PREZYGOTIC REPRODUCTIVE BARRIERS

The relative importance of different prezygotic barriers in this system varied. Geographic isolation alone may lead to RI of almost 90% for *E. albidum* and 70% for *E. mesochoreum*. The study species exhibited fine-scaled spatial segregation within contact zones. Indeed, at both YS and RC, *E. mesochoreum* was predominantly found near areas that historically had been tallgrass prairie (R.B. Kaul, G. Pollock & Crawford Co., IA Conservation Board, pers. comm.), suggesting that interspecific ecological differences are biologically based and that at least some of the geographic isolation could be ecological. However, we did not

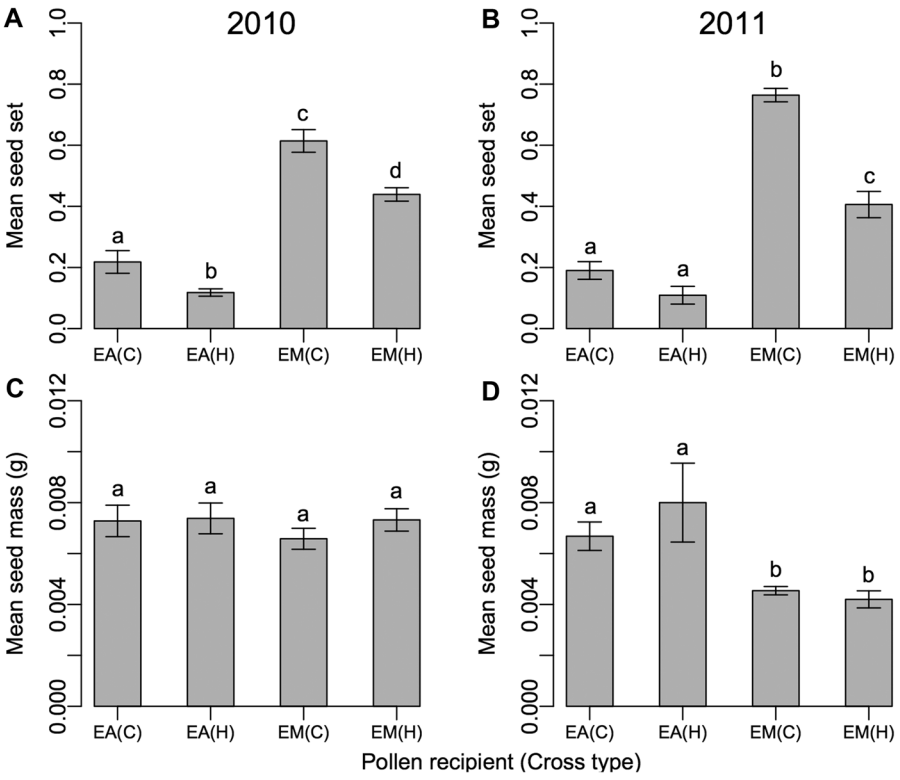

Figure 4. **(A, B) Mean seed set ± 1 standard error; and (C, D) Mean seed mass ± 1 standard error, from conspecific (C) and heterospecific (H) pollinations of *E. albidum* (EA) and *E. mesochoreum* (EM) in 2010 and 2011. Differing letters above the bars for each subfigure represent significant differences in mean seed set or mean seed mass at $P \leq 0.05$, based on planned comparisons after a significant main effect of cross type, in a one-way analysis of deviance.**

quantify whether these habitat preferences result from heritable variation in the ecological tolerances of these species, as would be the case with ecogeographic isolation. Extrinsic, historical factors are also likely to play a role in geographic isolation: extensive habitat loss and fragmentation in the United States Midwest has undoubtedly greatly reduced the opportunities for these species to come into contact, further complicating an understanding of the mechanisms underlying the strong geographic isolation of these species. Although they were segregated at contact zones, we chose not to quantify microspatial RI because spatial segregation only causes RI when it affects the extent of interspecific pollinator flights, and we did not record pollinator movements. It is important to consider that, even when geographic isolation is omitted, *E. albidum* and *E. mesochoreum* remain nearly completely reproductively isolated.

Our study indicates that flowering asynchrony plays a role in RI but that it is unlikely to be a consistently strong barrier to interspecific pollen transfer. After controlling for differences in regional climate, *E. mesochoreum* flowered significantly earlier than *E. albidum* across broad geographic and temporal scales, but flowering phenology still exhibited overlap. At the local scales at which pollination is relevant, patterns of flowering asynchrony varied between study years and were not always consistent with herbarium data. Considered in isolation, flowering asynchrony reduced the probability of interspecific gene flow by ∼20% for both species, averaged across both years. However, in 2010, we observed little asynchrony. In 2011, interspecific flowering asynchrony was greater, and was similar to the results obtained from the herbarium records. We established a greater number of quadrats, across a wider range of sites and environments, in 2011 versus 2010, which likely led to our observation of greater asynchrony in 2011. Soil temperature is likely a major cue governing *Erythronium* emergence and flowering (Risser and Cottam 1967; R. B. Kaul, pers. comm.). The additional quadrats in 2011 were near the bottoms of wooded ravines, which likely experience delayed soil warming. Sampling across sites with a wider range of abiotic conditions may have revealed greater flowering asynchrony in 2010.

Although shifts in the timing and duration of flowering can arise directly from genome duplication (Levin 1983; Soltis et al. 2003), we cannot determine whether the limited flowering asynchrony we observed resulted from genome duplication or arose during subsequent divergence. Ultimately, because flowering phenology at fine spatial scales varied among years and sites, the

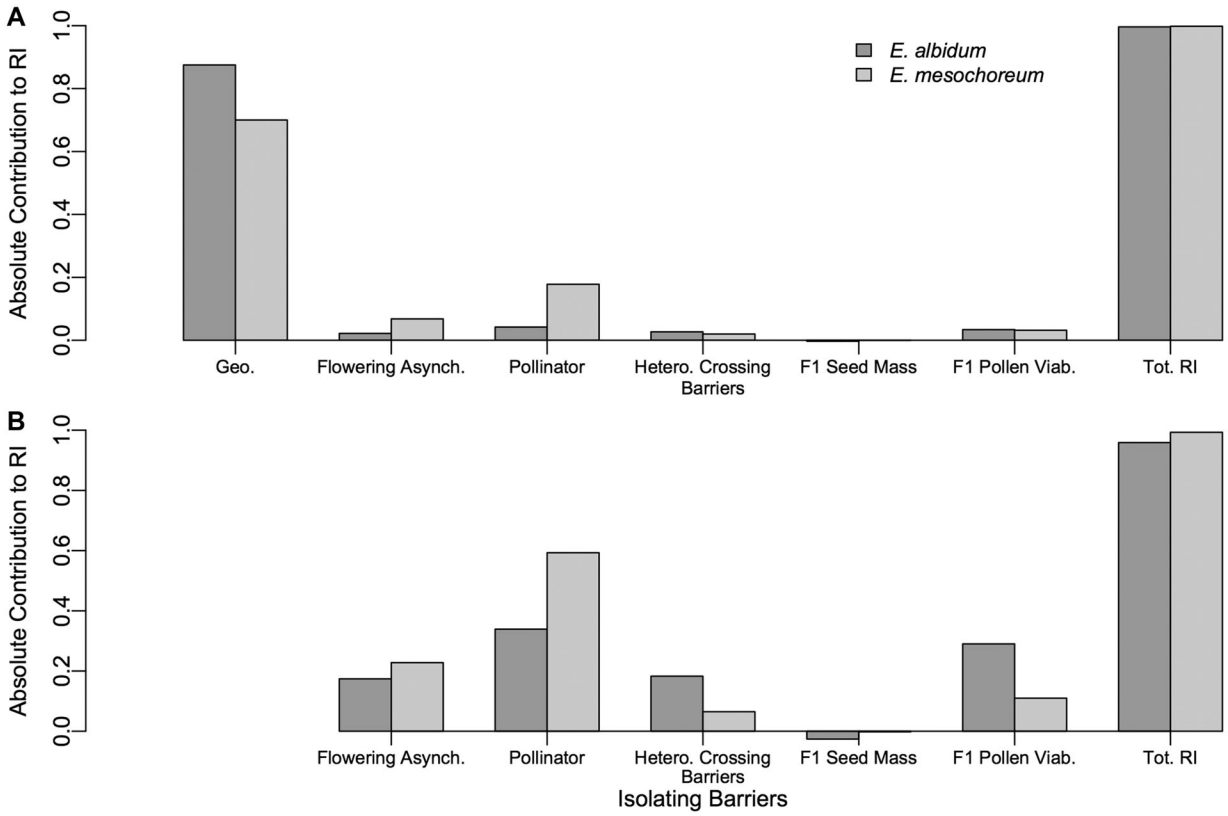

Figure 5. **The absolute contribution (AC) of each reproductive barrier to total reproductive isolation (RI) between *E. albidum* and *E. mesochoreum*, calculated both (A) with, and (B) without the contribution of geographic isolation. Total RI was calculated using the online supplement provided by Sobel and Chen (2014), based on their equation (4E). AC values were computed by first calculating total RI for the focal barrier and all preceding barriers (eqs. S2–S4), then subtracting from that value the total RI, including all preceding barriers, but excluding the focal barrier (Sobel and Chen 2014).**

potential for interspecific gene flow due to overlapping flowering is also likely to be locally variable.

The study species' floral visitor assemblages differed in species composition, indicating that even during periods of concurrent flowering, interspecific pollen transfer is likely limited.

Andrena carlini was the only abundant visitor captured on both plants, but it was more abundant on *E. albidum*, consistent with previous reports that *A. carlini* is the primary pollinator of this species (Schemske et al. 1978; Banks 1980). Because we also observed *A. carlini* on *E. mesochoreum*, the strength of pollinator-mediated RI was asymmetric, with *E. mesochoreum* experiencing greater isolation than *E. albidum*. The most abundant floral visitor of *E. mesochoreum*, *Andrena erythronii,* has previously been recorded infrequently on *E. albidum* (Schemske et al. 1978), but not in our study. Although we did not verify that the insects we captured were transferring pollen, previous workers have recorded *A. carlini* collecting pollen from *E. albidum* (Robertson 1929) and *A. erythronii* collecting pollen from *E. mesochoreum* (Michener and Rettenmeyer 1956; Krombein et al. 1979). Banks (1980) found that the pollen carried by two *A. carlini* specimens consisted of an average of 92% *Erythronium* pollen, and Michener and Rettemeyer (1956) hypothesized that *A. erythronii* likely cannot survive without *E. mesochoreum*, as it is one of the few species in bloom when this bee emerges. We are therefore confident that the primary visitors of *E. albidum* and *E. mesochoreum* were likely transporting pollen.

Site-specific differences in pollinator species pools may have magnified our observed differences in the floral visitor assemblages. However, MP and RC are close to one another and may share a common pollinator pool. BO was located approximately 30 km from these sites, but the majority of insects captured there belonged to species present at more than one other site. Our analyses using only the insects found at two or more sites yielded results consistent with the analyses of the full dataset, indicating that differences in the floral visitor assemblages of the study species are likely not simply due to site-specific differences.

Pollinator-mediated RI has been widely observed (Grant 1994; Coyne and Orr 2004). Closely related plants may have different pollinators (Ramsey et al. 2003; Moe and Weiblen

2012), and even shared pollinators may preferentially visit one taxon over the other, limiting interspecific pollen transfer (Fulton and Hodges 1999; Aldridge and Campbell 2007; Marques et al. 2012). Differences in flower morphology may also physically prevent interspecific pollination, even when pollinators are shared (Kay 2006). Despite recent interest in ecological mechanisms of speciation (Rundle and Nosil 2005; Schluter 2009), pollinator-mediated isolation has been examined in only a few mixed-ploidy taxa. Diploid and autotetraploid cytotypes of *Heuchera grossulariifolia* (Saxifragaceae) attract different pollinator assemblages, and shared pollinators demonstrate preferential visitation based on ploidy (Segraves and Thompson 1999; Thompson and Merg 2008). Pollinators of diploid and tetraploid *Chamerion angustifolium* (Onagraceae) exhibit foraging patterns that limit inter-cytotype flights, strengthening assortative mating (Kennedy et al. 2006). A limitation of our study is that we did not compare inter- versus intraspecific pollinator flights. Flowers of the study species are similar in size, shape, and color (Ireland 1957; Allen and Robertson 2002), but may differ in traits such as UV reflectance patterns or pollen and nectar production that could influence pollinator behavior. Further studies are needed to determine the mechanisms underlying the pollinator-mediated isolation we observed between these species.

POSTZYGOTIC REPRODUCTIVE BARRIERS

When considered sequentially, prezygotic barriers reduced the probability of interspecific gene flow dramatically, leaving fewer opportunities for heterospecific crossing barriers and hybrid inviability and sterility to limit interspecific gene flow. Triploid block is relatively common (Coyne and Orr 2004), and the reductions in heterospecific seed set that we observed may indeed have resulted from endosperm abnormalities associated with triploid block. Alternatively, reduced heterospecific seed set may have arisen from genic incompatibilities that accumulated sometime after genome duplication (Ramsey and Schemske 1998). Our evaluations of F1 seed mass indicate that when seeds are set, hybrid embryos may be well-provisioned for germination. Additionally, the presence of flowering hybrids at RC demonstrates that some hybrid seeds are viable and that hybrid plants can reach reproductive maturity. Whether hybrids suffer from reductions in germination or survivorship is unknown, and we were unable to compare hybrid and parental species germination directly. Our pollen staining study, while limited, indicates that male sterility may constrain hybrids from backcrossing with either parental species.

INTERSPECIFIC DIFFERENCES IN SELF-COMPATIBILITY

Self-incompatibility release, accompanied by increased selfing rates, can help neopolyploids overcome minority cytotype disadvantage and establish self-sustaining populations, thus fostering polyploid speciation. We failed to find conclusive evidence of self-incompatibility release in tetraploid *E. albidum*. Selfed seed set for *E. albidum* was higher than that of *E. mesochoreum* in 2011 only. Flowers of both species are protandrous and are provisioned with nectar (Banks 1980; Kaul 1989; K.R. pers. observation), traits that are typically associated with outcrossing. Although the pollen:ovule ratio of *E. mesochoreum* is not known, *E. albidum*'s pollen:ovule ovule ratio indicates that it is self-compatible but also adapted for outcrossing (Cruden 1977; Banks 1980). We did not measure self-fertilization rates for either species, but we have observed exserted stigmas of *E. albidum* in close contact with pollen-bearing anthers, such that selfing may occur (K.R., pers. observation). It is possible that some degree of self-compatibility could have facilitated divergence in this system, but the evidence is largely lacking.

Conclusion

Unlike allopatric speciation, in which RI arises via geographic isolation and subsequent selection, mutation, and drift, polyploid speciation necessitates that neopolyploids overcome minority cytotype disadvantage, generally by developing traits that foster assortative mating (Coyne and Orr 2004). Thus far, the role that prezygotic barriers play in this process has been largely overlooked. While our study alone cannot determine the traits responsible for speciation in this system, we add to a nascent body of literature indicating that prezygotic barriers such as geographic isolation, flowering asynchrony, and pollinator-mediated isolation can be important for maintaining reproductive isolation in mixed-ploidy systems. A wealth of previous work has demonstrated the evolutionary importance of triploid block and other physiological, postzygotic reproductive barriers for polyploid speciation and divergence, but to gain a fuller understanding polyploid speciation in angiosperms, future studies must also consider the role of earlier acting reproductive barriers.

ACKNOWLEDGMENTS

We thank Dr. Robert Kaul for sharing his *Erythronium* expertise and his thoughts on the potential for hybridization between these species, and we thank Rob Laport for comments on an earlier draft of this manuscript. Dr. Charles Kuszynski, Danielle Shea, and Zhi Hong Gill (Nebraska Center for Virology) and Dr. Eric Baack (Luther College) provided flow cytometry assistance. We thank the USDA-ARS Systematic Entomology Laboratory—Sam Droege (Hymenoptera identifications), Neal Evenhuis (Diptera identifications), Norman E. Woodley (Diptera identifications). This research was funded by the University of Nebraska's Center for Grassland Studies, School of Biological Sciences, and Center for Great Plains Studies.

DATA ARCHIVING

Data have been submitted to the Dryad Digital Repository. doi:10.5061/dryad.j4h5f.

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Associate Editor: D. Moeller
Handling Editor: R. Shaw

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Approximate geographical distributions of *Erythronium albidum* (dark gray shading) and *Erythronium mesochoreum* (light gray shading) in the United States.

Figure S2. Histogram of 334 flow cytometry relative fluorescence (RF) values for *E. albidum* (EA) and *E. mesochoreum* (EM) plants collected at contact zones in Nebraska, Kansas, and Iowa.

Figure S3. (A) *Erythronium albidum*; (**B**) *Erythronium mesochoreum*; (**C**) *E. albidum—E. mesochoreum* hybrids photographed at Red Cedar Recreation Area (Saunders Co., NE).

Figure S4. Mean seed set (± 1 standard error) for *Erythronium albidum* (EA) and *Erythronium mesochoreum* (EM) self-pollinations in 2010 and 2011.

Table S1. Herbarium sheets used in the analysis of broad-scale flowering asynchrony between *E. albidum* and *E. mesochoreum*.

Table S2. Nebraska sites containing *E. albidum* (EA), and/or *E. mesochoreum* (EM), used in the calculations of geographic isolation.

Table S3. Analysis of deviance table from the generalized linear models testing the effects of cross type, maternal species, and the cross type x maternal species interaction on *E. albidum* and *E. mesochoreum* percent seed set in 2010 and 2011.

Table S4. 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.