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Multiple reproductive barriers separate recently diverged sunflower ecotypes

Katherine L. Ostevik,[1,2] Rose L. Andrew,[3] Sarah P. Otto,[4] and Loren H. Rieseberg[1,5]

[1]*Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada*

[2]*E-mail: kate.ostevik@gmail.com*

[3]*School of Environmental and Rural Science, University of New England, Armidale, New South Wales, Australia*

[4]*Department of Zoology, University of British Columbia, Vancouver, British Columbia, Canada*

[5]*Department of Biology, Indiana University, Bloomington, Indiana*

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Measuring reproductive barriers between groups of organisms is an effective way to determine the traits and mechanisms that impede gene flow. However, to understand the ecological and evolutionary factors that drive speciation, it is important to distinguish between the barriers that arise early in the speciation process and those that arise after speciation is largely complete. In this article, we comprehensively test for reproductive isolation between recently diverged (<10,000 years bp) dune and nondune ecotypes of the prairie sunflower, *Helianthus petiolaris*. We find reproductive barriers acting at multiple stages of hybridization, including premating, postmating–prezygotic, and postzygotic barriers, despite the recent divergence. Barriers include extrinsic selection against immigrants and hybrids, a shift in pollinator assemblage, and postpollination assortative mating. Together, these data suggest that multiple barriers can be important for reducing gene flow in the earliest stages of speciation.

KEY WORDS: **Ecological divergence, *Helianthus petiolaris*, incipient speciation, pollen competition, reciprocal transplant, sand dune adaptation.**

To understand speciation, we must understand how and why groups of organisms stop exchanging genes. A direct approach to this problem is to characterize the traits and mechanisms impeding gene flow between diverging or recently diverged groups of organisms (Coyne and Orr 2004). Determining the order in which these reproductive barriers arise not only distinguishes between mechanisms that act early in speciation and those that act later, but it also provides clues regarding the processes underlying their evolution. Although reproductive barriers can arise via several processes including divergent natural selection (e.g., Rundle et al. 2000), reinforcement (e.g., Hopkins and Rausher 2012), the fixation of different alleles despite similar selection (e.g., Fishman and Willis 2006) and drift (e.g., Lynch and Force 2000), the action and importance of these processes depend on specific ecological and evolutionary conditions. For example, similar selection is unlikely to fix different alleles in groups connected by gene flow (Schluter 2009) while reinforcement cannot act if hybridization has ceased (Butlin 1989; Rundle and Schluter 1998). Because reproductive barriers affect conditions such as gene flow and hybridization, identifying the order in which they arise can point to the ultimate causes of reproductive isolation (RI) between species.

Reproductive barriers are grouped into three categories: premating (e.g., flowering time), postmating–prezygotic (e.g., conspecific pollen precedence), and postzygotic (e.g., hybrid sterility). Many studies have found that prezygotic barriers have stronger effects than postzygotic barriers among closely related taxa (Lowry et al. 2008a; Dell'Olivo et al. 2011; Sánchez-Guillén and Wellenreuther 2012; Sobel and Streisfeld 2015), especially considering that prezygotic barriers act first. However, others have found that postzygotic barriers (Kozak et al. 2012) or both types of barriers are important for reducing gene flow (Sambatti et al. 2012; Scopece et al. 2013; Briscoe Runquist et al. 2014; Kao et al. 2015). Reproductive barriers are also categorized by whether the environment is necessary for their action, where extrinsic reproductive barriers are environmentally dependent and intrinsic reproductive barriers are not. This distinction has been emphasized

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Table 1. Mean weight of the seeds produced by dune and nondune plants grown in a common garden.

Ecotype	N	Seed weight (mg)	95% CI
Dune	41	11.3	10.5–12.1
Nondune	24	5.0	4.5–5.6

for postzygotic barriers because hybrids can face strong selection under natural conditions and yet thrive under controlled conditions.

Observing the accumulation of reproductive barriers directly is rarely feasible. However, it is possible to infer likely sequences of barrier evolution by measuring barriers across systems that span the stages of speciation (Coyne and Orr 2004). Although there are excellent examples of multiple barriers measured between well-established species (e.g., McMillan et al. 1997; Ramsey et al. 2003; Kay 2006; Martin and Willis 2007; Matsubayashi and Katakura 2009; Dell'Olivo et al. 2011) and some at intermediate stages of speciation (e.g., Aston 1966; Antonovics 1968; McNeilly 1968; McNeilly and Antonovics 1968; Nosil 2007; Lowry et al. 2008b; Briscoe Runquist et al. 2014; Melo et al. 2014; Sobel and Streisfeld 2015), the success of this method relies on numerous studies spread throughout the speciation continuum. In particular, we need more studies that identify and measure barriers at the earliest stage of divergence, as these barriers arguably drive the speciation process (rather than accumulate after speciation is mostly complete, Coyne and Orr 2004).

The prairie sunflower *Helianthus petiolaris* is a good system for measuring RI early in the speciation process. Although complete speciation is not inevitable, we consider dune and nondune ecotypes within this sunflower to be incipient species. Divergence between typical nondune *H. petiolaris* and a sand dune ecotype found in Colorado began less than 10,000 years ago (Andrew et al. 2013). Gene flow between the two types is asymmetric (with fewer immigrants from the surrounding sand sheet into the sand dune environment) and too high for drift to cause widespread differentiation (Andrew et al., 2012, 2013). But gene flow is too low to prevent some adaptive divergence, and consequently, there are a few large regions of genomic divergence that differentiate the ecotypes (Andrew and Rieseberg 2013). Also, related work on additional species pairs within *Helianthus* (Rieseberg et al. 1995; Sambatti and Rice 2006; Yatabe et al. 2007; Raduski et al. 2010; Sambatti et al. 2012; Renaut et al. 2013) means that this study will provide insight into variation in the development of RI among closely related taxa.

In this article, we comprehensively test for RI between dune and nondune ecotypes of *H. petiolaris*. We measure selection against immigrants and hybrids, flowering time differences, pollinator assemblages, postpollination assortative mating, hybrid inviability, and hybrid sterility. We ask whether reproductive barriers are limited to those associated with local adaptation or whether additional barriers separate these recently diverged ecotypes.

Methods

STUDY SYSTEM AND SEED COLLECTIONS

Helianthus petiolaris is a widespread sunflower species that is annual, self-incompatible, and hermaphroditic. It is typically found in sandy soils across the central United States (Heiser et al. 1969). However, populations of *H. petiolaris* inhabit active sand dunes at Great Sand Dunes National Park and Preserve (GSD) in Colorado. These dunes are the tallest in North America and are a challenging environment for plant species due to shifting sand and low soil nutrients (Johnson 1968; Morenno-Casasola 1986; Andrew et al. 2012). *Helianthus petiolaris* plants found on dunes are morphologically distinct from typical *H. petiolaris* plants found on the vegetated sand sheet surrounding the dunes (Andrew et al. 2012). Most strikingly, dune seeds are more than two times heavier than nondune seeds, and these differences are maintained in a common garden (Table 1, Supporting Information Methods and Results).

There are several places in the life cycle of a plant where reproductive barriers could disrupt hybridization between two ecotypes. In order for hybridization to occur, pollen from one ecotype must arrive on the stigma of the other ecotype. This could occur because pollinators travel between the two habitat types or because both ecotypes survive in the same habitat where pollinators move among them. The first method of pollen transfer can be disrupted by the ecotypes flowering at different times (flowering time) or by each habitat having unique pollinators (pollinator assemblages). The second method of pollen transfer can be disrupted by mismatched ecotypes failing to survive or reproduce in alternate habitats (selection against immigrants), the ecotypes flowering at different times even when grown together (flowering time), or by pollinators favoring movements between plants of the same ecotype (pollinator constancy). If pollen does arrive on the stigma of a mismatched ecotype, hybridization could still be disrupted if the pollen fails to fertilize ovules (postpollination assortative mating). Finally, if fertilization does occur, hybrid seeds may not be viable (hybrid inviability), may fail to reproduce (hybrid sterility), or may have poor fitness under natural conditions (extrinsic selection against hybrids).

Throughout this study, we used seeds collected from natural dune and nondune populations and greenhouse generated hybrid seeds to measure reproductive barriers in *H. petiolaris* (see Table S1 and Fig. S1 for details). We generated two F_1 seed lots by pooling crosses made between multiple dune and nondune individuals from several populations. One seed lot was produced using dune maternal plants (F_1D) and the other using nondune

maternal plants (F_1N). We also generated two types of backcrosses by pooling crosses made between maternal F_1s (equal numbers of F_1D and F_1N) and either dune (BCD) or nondune (BCN) pollen donors from several populations. The result was a single genetically variable seed lot for each of four hybrid types.

Because seed size is often determined by maternal genotype (Li and Li 2015) and can affect subsequent phenotypes and fitness (Westoby et al. 1992), we were interested in the relationship between seed type and weight. Therefore, we weighed 90 groups of 10 seeds from three dune populations (D1–D3), three nondune populations (N1–N3), and four hybrid (F_1D, F_1N, BCD, BCN) seed types and used ANOVA with a Welch correction and a post hoc Games-Howell test (posthocTGH function, userfriendlypackage, Peters 2015) to test for different seed weights. Throughout this study, we used R version 3.1.3 (R Core Team 2015) with the library plyr (Wickham 2011) for our statistical analyses.

SELECTION AGAINST IMMIGRANTS AND HYBRIDS

To test for selection against immigrant and hybrids, we reciprocally planted the seeds weighed above into a site in the dunes and a site on the sand sheet (Table S1, Fig. S1). At each site, we established nine ring-shaped plots along each of five transects drawn through a natural population of sunflowers. We cleared each plot of vegetation and divided the plots radially into 16 equal subsections. After storing seeds at 4°C on wet filter paper for four weeks to mimic overwintering conditions (Donovan et al. 2010), we randomly assigned the 10 seed types to subsections and planted 10 seeds of the assigned type 5 cm deep in each subsection. To determine the number of naturally recruited seedlings in each plot (volunteers), we made 5 cm holes in the remaining six subsections, later referred to as control subplots, without planting seeds (see, e.g., Donovan et al. 2010). We counted the number of seedlings in each subplot after six weeks, and we recorded the number of surviving seedlings and their heights after an additional eight weeks. Once the plants began flowering, we recorded the number of flower heads with open disc florets every five to seven days, covered pollinated flower heads to prevent seed loss, collected mature flower heads, and counted and weighed seeds.

In our analysis of seedling emergence, we fit generalized linear mixed models (GLMMs) to the number of seedlings that emerged in each subplot. These models assumed a Poisson distribution of error terms, accounted for frequent zero-valued observations (zero-inflation), and showed no evidence of additional overdispersion (glmmADMB function and package; Fournier et al. 2012; Skaug et al. 2014). We fit models with environment, type (10 seed types and control), and their interaction as fixed effects and plot as a random effect. We identified significant fixed effects by comparing nested models using likelihood ratio tests. In addition, we used GLMMs as described above to look for a relationship between seed weight and seedling emergence and to look for differences between dune and nondune emergence in isolation while including population as a random effect.

Although emergence in the control subplots was very rare (only 13 seedlings emerged in 540 control subplots), there could be some natural recruitment in the experimental subplots. We looked for these volunteers by examining the weight of seeds that each plant produced (seed size differences are maintained in common gardens). All but three plants produced seeds that were the expected weight (Fig. S2), and our results were unaffected by whether these plants were included or excluded in analyses (we present the former). We do not include seedlings that emerged in control subplots in the remaining analyses.

We used linear mixed models (lmer function; lme4 package; Bates et al. 2015a,b) to explore the effects of type, environment, and their interaction on three proxies of fecundity: (1) plant height, (2) total number of flower heads produced, and (3) total number of seeds produced. Because of low emergence and survival during our reciprocal transplant, we grouped plants across the populations and hybrid types to make three composite types (dune, hybrid, and nondune) for this analysis. We used the mean trait value of surviving plants in each subplot as the response variable and included plot as a random effect. Again, we compared nested models with likelihood ratio tests to determine the significance of fixed effects.

To analyze total fitness over the life cycle, we used ASTER, a likelihood method for fitting multiple fitness components with different probability distributions in a single model (Geyer 2007). We combined three sequential components of fitness: (1) emergence, (2) seedling survival, and (3) number of seeds produced (aster function and package; Geyer 2015). We included plot and subplot as random effects and tested seed type, environment, and their interaction as fixed effects using likelihood ratio tests. Like the emergence data, we repeated this analysis using only dune and nondune types and including a population random effect. Also, because sunflowers are hermaphroditic, we repeated this analysis using total number of flower heads in addition to total seed number to better account for both male and female contributions to fitness. The results of this analysis were comparable (Tables S7–S10), so we present the first analysis only.

FLOWERING TIME

We tested whether day to first flower was affected by seed type, environment, and their interaction for plants that survived in our reciprocal transplant using an interval censored survival analysis (survfit and survreg functions; survival package; Therneau and Grambsch 2000; Therneau 2015). As in the fecundity analyses, we grouped populations and hybrid types into three composite types (dune, hybrid, and nondune) and used mean values for each subplot as the response. Survival was modeled using the logistic distribution, and we included plot as a random effect.

To measure flowering time in natural populations, we positioned time-lapse cameras in three dune and three nondune populations (Table S1). Each camera photographed a patch of naturally occurring sunflowers daily throughout the flowering period. We used ImageJ (Schneider et al. 2012) to count the number of open flower heads visible in a static section of the population every day. When poor light conditions affected our ability to count open heads (<2% of photos), we used the average number of open heads on adjacent days to estimate the missing data. For each environment, we added the number of open heads across sites for each day and divided by the total number of open heads across all days. This resulted in a distribution of the fraction of heads that were open on any given day for the dune and sand sheet environments.

POLLINATOR ASSEMBLAGES

To assess whether the assemblages of potential pollinators differed between dune and sand sheet environments, we netted insects visiting the natural sunflowers surrounding the dune and nondune reciprocal transplant sites approximately once per week (Table S2). This component of RI measures the degree to which habitat isolation limits pollen flow between environments. It does not assess pollen flow between different ecotypes in the same environment (pollinator constancy). We did not measure pollinator constancy in this study because dune and nondune sunflowers have similar floral morphology, do not have a single characteristic pollinator, and are typically visited by many insect species. We collected insects that we observed touching mature sexual parts of sunflowers at the dune and nondune sites during the same two-hour window on consecutive days and made the next pair of collections during a different time period in the following week. This sampling method allowed the collections at the two sites to be paired in time, but the paired collections to be spread throughout the flowering period and time of day, with the goal of sampling as many potential pollinators as possible. To sample potential pollinator assemblages more broadly, we also positioned malaise traps and pan traps at several dune and nondune populations (Table S2). We sorted the collections into morphospecies and sent bees and nonparasitoid wasps (Aculeate specimens) to the USDA ARS Bee Biology & Systematics Laboratory for identification.

We tested whether potential pollinator assemblages differed between the environments after controlling for distance using partial Mantel tests. We reduced our data from all collection methods to the most likely pollinators (Hymenoptera, Lepidoptera, Diptera, and Coleoptera) and calculated a Bray–Curtis dissimilarity index (Bray and Curtis 1957; mantel.partial and vegdist functions; vegan package; Oksanen et al. 2015). The effect size and significance of the partial Mantel test was not sensitive to the use of alternative dissimilarity indices (e.g., Mountford 1962).

POSTPOLLINATION ASSORTATIVE MATING

We used plants grown from wild seed in the greenhouse to create three pollen types: pooled dune pollen, pooled nondune pollen, and a 50:50 mixture of the ecotypes. We made pollen pools by combining equal volumes of pollen from at least three plants from at least three populations within an ecotype. Dune and nondune pollen donors were homozygous for alternate alleles of a genetic marker (PCO_08) strongly associated with ecotype. The restriction enzyme, *AvrII*, cleaves DNA fragments with the allelic variant common on the dunes but not the variant common on the sand sheet. We made pollen mixtures by combining equal weights of pooled dune and pooled nondune pollen. A test of this method showed that equal weights of pollen corresponded to indistinguishable numbers of pollen grains.

To quantify postpollination assortative mating, we covered immature flower heads with mesh to prevent natural pollination and hand pollinated each head with a pollen mixture to which the maternal plant had not contributed (pollen competition experiment). There was no need to emasculate flowers because *H. petiolaris* is self-incompatible. We then extracted DNA from 1 to 23 (mean = 6.2) seeds produced by each maternal plant and determined seed paternity with marker PCO_08. When we could not definitively assign a paternal ecotype to a seed (i.e., when the maternal plant and the seed were heterozygous at marker PCO-08), we excluded that seed from our analysis (assigning paternity using a likelihood approach yielded virtually identical results). In total, we genotyped 1528 seeds (1376 of which were informative) and assayed 116 maternal plants from 11 dune and eight nondune populations (Table S1) for assortative mating.

We analyzed the data from the pollen competition experiment using logistic regression and assessed the effect of maternal ecotype on paternal ecotype using likelihood ratio tests (glmer function; lme4 package; Bates et al. 2015a,b). The logistic models used the binomial link function; included pollen mixture, maternal plant, and maternal population as random effects; and showed no evidence of overdispersion.

For a subset (68) of the plants that received pollen mixtures, we pollinated two additional flower heads to assess cross-ecotype seed production, one with pooled dune pollen and the other with pooled nondune pollen made as described above (seed set experiment). In these cases, we pollinated 30 receptive styles and counted the number of seeds produced to determine whether the pollen types had biased success in the absence of direct competition. We simplified the resulting paired data by subtracting the number of seeds produced after nondune pollination from those produced after dune pollination for each plant. Then we used one-sample *t*-tests to determine whether the differences departed significantly from 0 for each ecotype.

Finally, we pollinated several dune flower heads with both pooled pollen types in sequence to evaluate the effect of timing

on pollination success (pollination timing experiment). We varied which pollen type was used first (D-N and N-D) and the time between pollinations (6 or 12 hours), for a total of four treatments. We tested whether the time between pollinations changed the siring success of dune pollen when flowers were pollinated with dune pollen before nondune pollen using logistic regression as described in the pollen competition analysis above. We repeated this analysis for the flowers in which nondune pollen was applied before dune.

INTRINSIC HYBRID INVIABILITY

We used the seeds produced in the seed set experiment described above (three seeds fathered by dune plants and three seeds fathered by nondune plants for each maternal plant) to test hybrid seed germination. Specifically, we scarified seeds, soaked them in a 100 mg/L solution of gibberillic acid for one hour to break dormancy (Chandler and Jan 1985), and put the seeds on wet filter paper. We put the seeds in the dark for two days, removed their seed coats, replaced the filter paper, and returned them to the dark. We moistened the filter paper and checked for germination (radicles $>$ 2 mm) every 2 days. After 4 days, 98% of the seeds had germinated and we terminated the experiment 10 days later. To explore the potential effects of seed dormancy, we repeated the basic experiment without a gibberillic acid treatment. The results of that experiment were similar (Supporting Information Methods and Results).

We used GLMMs to determine whether germination was explained by maternal ecotype, paternal ecotype, and/or their interaction (glmer function; lme4 package; Bates et al. 2015a,b). Similarly, we compared models with and without the explanatory variable, cross type (hybrid versus pure). In both cases, we modeled germination using a binomial link function and included maternal population and pollen mixture as random effects. The models showed no evidence of overdispersion.

HYBRID STERILITY

We made crosses within and between dune and nondune ecotypes, supplementing the nondune parents collected within GSD with *H. petiolaris* individuals collected in New Mexico by the U.S. Department of Agriculture (USDA). We grew 8–12 progeny from each cross type under controlled greenhouse conditions and collected their pollen. As a proxy for pollen viability (Nepi and Franchi 2000), we stained the pollen in 30% (w/v) sucrose and 0.1% (w/v) thiazolyl blue tetrazolium bromide (MTT) stain for one day (24 hours; Chandler et al. 1986). We arcsine transformed the proportion of stained grains (180–1000 grains scored/plant) and determined whether cross type (within or between ecotype) significantly predicted pollen viability. Pollen viability was not strongly affected by the two nondune types (GSD vs. USDA; $\chi^2_2 = 0.06$, $P = 0.94$); we therefore combined all nondune parents for the purpose of calculating barrier strength.

CALCULATING BARRIER STRENGTH

Following Sobel and Chen (2014), we use a method for calculating RI that yields a simple linear relationship between RI and the reduction of gene flow between groups:

$$\mathrm{RI} = 1 - 2^* \left(\frac{H}{H + C}\right) \quad (1)$$

where H and C represent either the number of heterospecific and conspecific (in this case between and within ecotype) matings or the number of viable heterospecific and conspecific offspring given equal opportunities for both. Using this equation, the RI values 1, 0, and –1 correspond to 0, 50, and 100% heterospecific outcomes, respectively. When there were not equal opportunities for conspecific and heterospecific outcomes, we scaled H and C by the opportunity for each. In addition, we calculated the strength of RI that reduces gene flow from the sand sheet into the dunes ($\mathrm{RI_{N\to D}}$) separately from the strength of RI that reduces gene flow from the dunes into the sand sheet ($\mathrm{RI_{D\to N}}$) whenever possible.

We used equation (1) to calculate barrier strengths for selection against immigrants and hybrids, postpollination assortative mating, intrinsic hybrid inviability, and hybrid sterility. For example, to calculate $\mathrm{RI_{N\to D}}$ caused by selection against $\mathrm{F_1}$ hybrids, we used the number of seeds produced by $\mathrm{F_1}$ hybrid plants in the dune environment divided by 0.2 as H (2/10 of the seeds types were $\mathrm{F_1}$s) and the number of seeds produced by dune plants in the dune environment divided by 0.3 as C (3/10 of the seeds types were D1–D3). In the other cases, we calculated barrier strength using the total number of seeds produced by local and immigrant plants during our reciprocal transplant (selection against immigrants), the number of hybrid and parental seeds produced after mixed pollinations (postpollination assortative mating), the proportion of hybrid and parental seeds types that germinated (hybrid inviability), and the average pollen viabilities of hybrid and parental plants (hybrid sterility).

When reproductive barriers are based on temporal or spatial co-occurrence (flowering time and pollinator assemblages), the minimum value of RI is zero (complete co-occurrence), so, we use a comparable equation for RI that ranges from 0 to 1:

$$\mathrm{RI} = 1 - \left(\frac{S}{S + U}\right), \quad (2)$$

where S and U are the shared and unshared portions of occurrence (Sobel and Chen 2014). In these cases, we calculated RI using the proportion of dune and nondune flowering distributions estimated by time-lapse photography that were shared and

unshared (flowering time) and the number of pollinators caught visiting the sunflowers that were shared between environments or found in a single environment (pollinator assemblages).

For each barrier strength calculation, we used 10,000 bootstrap samples of our raw datasets to calculate 95% confidence intervals (Efron 1987). If a bootstrap replicate sampled 0 values for both H and C, we considered barrier strength to be 0, yielding a conservative confidence interval that was more likely to overlap 0.

To calculate total RI, we used Sobel and Chen's (2014) equation 4E, which considers shared and unshared space and time (barriers that affect co-occurrence) before other types of barriers. Estimates of total RI assume that reproductive barriers act sequentially. In plants, however, the initial source of genetic exchange can be from seed or pollen flow and some barriers affect only one source (Sambatti et al. 2012). For example, immigrant inviability, which is the reduced survival of foreign individuals (in this case seeds) in a habitat, only affects seed flow while differences in pollinator assemblages only affect pollen flow. For this reason, we calculated total RI for pollen and seed flow separately (see Table S3 for a list of the barriers included in each calculation). Finally, we resampled from the distributions of each individual barrier strength 10,000 times during total RI calculation to determine 95% confidence intervals for the estimates.

Results

We found that seed weight was strongly related to seed type ($F_{5,308} = 2359$, $P < 0.001$, Fig. 1), with all pairwise comparisons between types being significantly different (all $t_{df}>5.5$, where df > 125, all $P < 0.001$) except between D2 and F_1D ($t_{168} = 0.35$, $P = 0.99$). F_1 seeds produced by dune mothers (F_1D) weighed roughly the same as dune seeds, and F_1 seeds produced by nondune mothers (F_1N) weighed roughly the same as nondune seeds (Fig. 1). Both backcrosses (produced by F_1 mothers) had intermediate seed weights, though seeds with dune fathers (BCD) were slightly heavier than seeds with nondune fathers (BCN; Fig. 1).

SELECTION AGAINST IMMIGRANTS AND HYBRIDS

During the reciprocal transplant, more seedlings emerged in all treatment subplots relative to unseeded control subplots (Figs. 1, S3; Table S5).

When we consider only dune and nondune treatments, more seedlings emerged in the dune environment ($\chi^2_1 = 19.85$, $P < 0.001$), and more dune seedlings emerged than nondune seedlings in both environments ($\chi^2_1 = 20.82$, $P < 0.001$; Figs. 1, S3; Table S4). We found no evidence of an environment-by-type interaction ($\chi^2_1 = 0.74$, $P = 0.39$). On the other hand, nondune plants had higher fecundity than dune plants in the sand sheet environment, while there was little effect of ecotype on fecundity in the dune environment (Fig. 2, Table S6). Consequently, we found significant type-by-environment interactions for each proxy of fecundity (plant height: $\chi^2_2 = 13.41$, $P = 0.001$; number of flower heads: $\chi^2_2 = 37.8$, $P < 0.001$; seed number: $\chi^2_2 = 6.97$, $P = 0.03$). We also found significant environment-by-type interactions in our ASTER models, whereby each ecotype was the most fit in the environment with which it is associated ($\chi^2_1 = 1951.2$, $P < 0.001$, Table S7).

The emergence patterns of all seeds types (D1–D3, F_1D, BCD, BCN, F_1N, N1–N3) in the reciprocal transplant parallels those of the parental ecotypes; more seedlings emerged in the dune environment ($\chi^2_1 = 19.37$, $P<0.0001$), the different seed types emerged at different rates ($\chi^2_1 = 337.51$, $P<0.0001$), and there was no evidence of an environment-by-type interaction ($\chi^2_1 = 14.54$, $P = 0.1497$, Figs. 1, S3; Table S5). In addition, seedling emergence was significantly associated with seed weight ($\chi^2_1 = 118.68$, $P<0.001$), although weight did not explain as much variation as seed type ($\Delta AIC = +43$). This is illustrated in the emergence patterns of the four hybrid cross types (Figs. 1, S3). The heavier F_1D seedlings emerged better than F_1N seedlings, but BCD seedlings also emerged better than BCN seedlings even though they have similar weights. In general, the more dune-like hybrids (F_1D and BCD) emerged well, though not as well as dune seeds, while the other hybrids emerged poorly (Figs. 1, S3). The fecundity of hybrids was consistently similar to the less fecund dune plants. Thus, while nondune plants were taller, had more flowers, and produced more seeds on the sand sheet, the hybrids did not exhibit any advantage (Fig. 2). In the full ASTER analysis, we found significant environment-by-type interactions ($\chi^2_9 = 137.6$, $P < 0.001$, Tables S8) in which the three dune populations were the most fit in the dune environment and two of the three nondune populations were the most fit in the sand sheet environment, compared to any of the other hybrid or parental types (Fig. S4). Essentially, local ecotypes tended to be more fit than the hybrids and the mismatched ecotype.

This local adaptation resulted in substantial RI between the ecotypes. Gene flow into the dune environment is strongly impeded by selection against immigrants ($RI_{N\to D} = 0.89$), particularly at the seedling emergence stage, and by selection against each hybrid type ($RI_{N\to D}$: F_1D = 0.44, BCD = 0.77, BCN = 0.94, F_1N = 0.99, Table 2, Fig. 3). Gene flow on to the sand sheet environment is likely hindered by selection against immigrants ($RI_{D\to N} = 0.76$), particularly at the fertility stage, and by selection against the hybrid types ($RI_{D\to N}$: F_1D = 0.43; BCD = 0.74, BCN = 0.55, F_1N = 1), although the confidence intervals estimated by bootstrapping were very broad (Table 2, Fig. 3). This occurred because emergence was so low in the sand sheet environment that there was substantial variation among bootstraps in whether any seedlings were sampled.

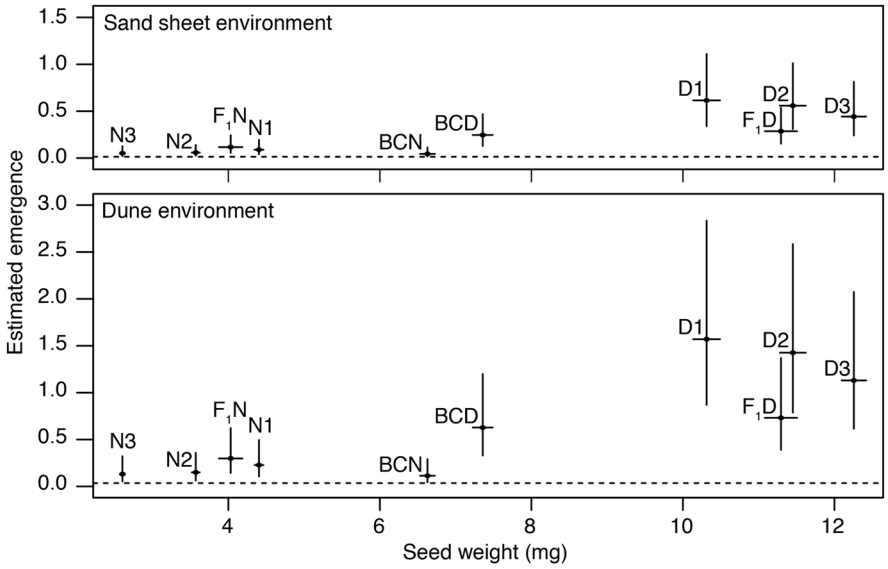

Figure 1. Mean number of seedlings that emerged in each plot subsection (estimated by GLMM) versus mean seed weight for each type. Emergence data should be interpreted relative to the planting of 10 seeds in each subplot, except the controls, and with the possibility of natural seedling recruitment in mind. Seed type had a significant effect on seedling emergence in both environments ($\chi^2_1 = 337.51$, $P < 0.0001$), and overall seedling emergence was better in the dune environment ($\chi^2_1 = 19.37$, $P < 0.0001$). However, there was no environment-by-seed type interaction ($\chi^2_1 = 14.54$, $P = 0.1497$). The dotted line represents the upper confidence limit for emergence in the control subplots and the error bars are 95% confidence intervals. N1–N3, nondune populations; F_1N, F_1 seeds with nondune mothers; BCN, F_1 plants backcrossed to nondune plants; BCD, F_1 plants backcrossed to dune plants; F_1D, F_1 plants with dune mothers; D1–D3, dune populations.

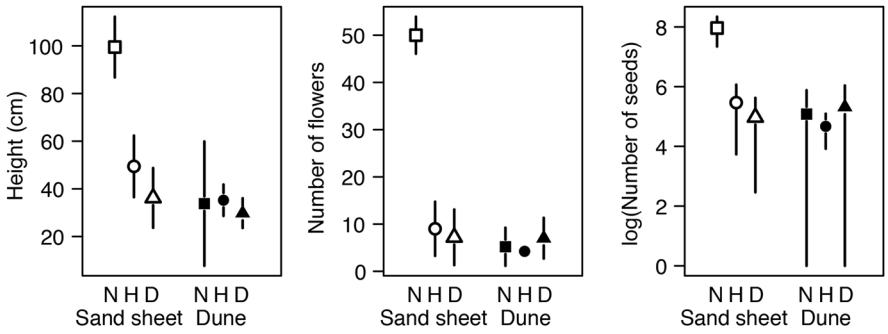

Figure 2. Mean values of three proxies of fecundity (height, number of flowers, and number of seeds) measured for nondune (N, squares), hybrid (H, circles), and dune (D, triangles) plants grown in sand sheet (open symbols) and dune (solid symbols) environments ($N = 2, 7, 6, 5, 24, 87$ from left to right in each plot). There are significant environment-by-type interactions for each proxy (plant height: $\chi^2_2 = 13.41$, $P = 0.001$; flower number: $\chi^2_2 = 37.8$, $P < 0.001$; seed number: $\chi^2_2 = 6.97$, $P = 0.03$). The error bars represent 95% confidence intervals.

FLOWERING TIME

There was no effect of environment, type, or an environment-by-type interaction on the day to first flower in the data from the reciprocal transplant ($\chi^2_1 = 1.40$, $P = 0.24$; $\chi^2_2 = 4.82$, $P = 0.09$; $\chi^2_{1.4} = 1.81$, $P = 0.26$; Fig. S5). Nevertheless, imperfect overlap between dune and nondune flowering distributions estimated from time-lapse photography (Figs. 4, S6) generated a barrier strength of 0.093 (Table 2, Fig. 3), with dune plants flowering slightly earlier than nondune plants. Because we used density distributions and any overlap is an equal proportion of each distribution, these estimates of RI are necessarily symmetrical ($RI_{N\to D} = RI_{D\to N}$). This result must be treated cautiously because chance fluctuations in the measurement of flowering distributions could lead to an apparent RI, even if none were present. To assess this possibility, we reestimated RI using two samples drawn with replacement from a single flowering time distribution (either dune or nondune). The

Table 2. Reproductive barrier strengths (95% confidence intervals based on 10,000 bootstrap replicates).

Reproductive barrier	$RI_{N\rightarrow D}$	$RI_{D\rightarrow N}$
Selection against immigrants	0.89 (0.66, 0.99)	0.76 (–1, 0.98)
Flowering time	0.093 (0.09, 0.11)	0.093 (0.09, 0.11)
Pollinator assemblages	0.55 (0.48, 0.63)	0.36 (0.30, 0.41)
Postpollination assortative mating	0.38 (0.32, 0.45)	0.12 (0.04, 0.20)
Intrinsic F_1 hybrid inviability	0.003 (–0.01, 0.01)	0.006 (0, 0.16)
Selection against F_1D hybrids	0.44 (–0.02, 0.79)	0.43 (–1, 1)
Selection against F_1N hybrids	0.99 (0.97, 1)	1 (0–1)
F_1 hybrid pollen sterility	0.01 (–0.02, 0.05)	–0.03 (–0.08, 0.01)
Selection against BCD	0.77 (0.48, 0.92)	0.74 (–1, 1)
Selection against BCN	0.94 (0.78, 1)	0.55 (–1, 1)
Total RI (seed flow)	0.999 (0.972–0.999)	0.992 (–1, 1)
Total RI (pollen flow)	0.986 (0.925–0.998)	1 (–1, 1)

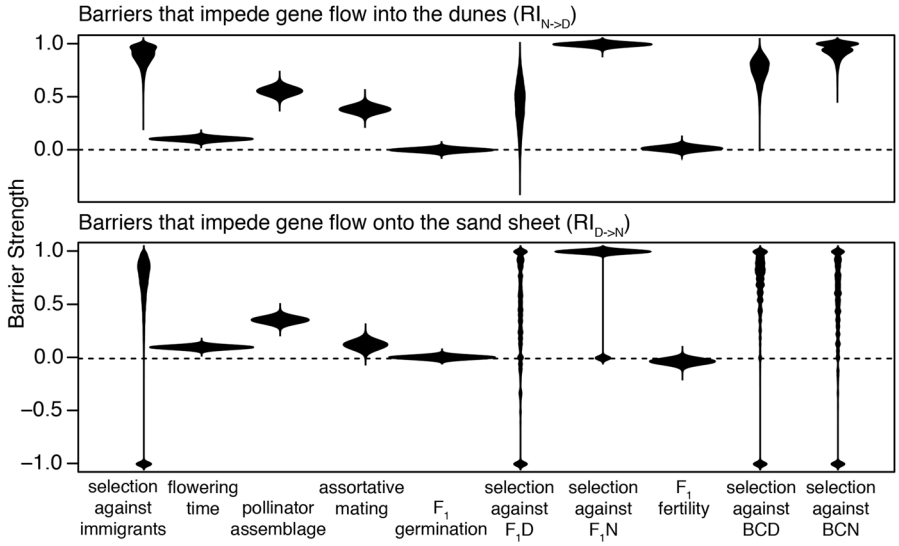

Figure 3. The distributions of barrier strength estimates from 10,000 bootstrap replicates (*beanplots*). The thickness of each bean is proportional to the number of bootstrap replicates consistent with a given barrier strength. The barrier strength caused by differences in flowering time is symmetrical (the two beans are identical). Reproductive barrier strengths greater than zero (dashed line) retard gene flow and those less than zero facilitate gene flow. The strongly negative barrier strengths are a result of bootstrap replicates that sampled immigrant/hybrid plants that produced some seed but failed to sample local plants that produced seed (i.e., immigrant/hybrid plants had much higher fitness than local plants).

resulting barrier strength approximately halved (0.035–0.053), indicating that the observed RI is about 0.044 higher than expected based on sampling effects alone.

POLLINATOR ASSEMBLAGE

There was a significant difference between potential pollinator assemblages collected in each environment after accounting for the distances between sites ($r = 0.35$, $P = 0.001$, Fig. S7). There were several likely pollinators (sampled visiting sunflowers at least 30% of the time) that showed particularly biased distributions (Table S11). For example, we collected 61 *Microbemex monodonata* specimens across six samples from the dune environment but only two individuals on the sand sheet. Similarly, we collected 38 *Perdita dolichocephala* individuals across eight sand sheet samples and none in the dunes. When we consider the proportion of visits made by insects found in both environments (netted collections only), the strength of RI from nondune to dune is 0.55 and from dune to nondune is 0.36 (Table 2, Fig. 3).

POSTPOLLINATION ASSORTATIVE MATING

Following pollination by equal mixtures of dune and nondune pollen, dune pollen sired 66–72% of seeds produced by dune plants, and nondune pollen sired 52–60% of seeds produced by nondune plants ($\chi^2_1 = 24.4$, $P < 0.0001$, Fig. 5, Table S12). This assortative mating results in a barrier strength of 0.38 from nondune to dune and 0.12 in the other direction (Table 2, Fig. 3).

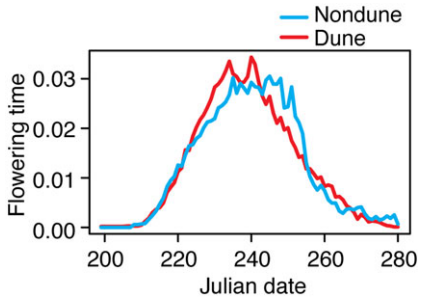

Figure 4. Composite flowering time distributions measured by time-lapse cameras in three dune and two nondune populations.

In the absence of pollen competition, nondune plants produced equivalent numbers of seeds after pollination with dune versus nondune pollen (mean difference 95% CI = –7.4–3.8, t_{23} = –0.66, P = 0.51, Fig. S8), while dune plants produced more seeds after pollination with dune pollen (mean difference 95% CI = 1.0–7.6, t_{40} = 2.64, P = 0.01, Fig. S8). Given the success of dune and nondune pollen on dune plants, we would expect 54–59% of seeds to be sired by dune plants after mixed pollination. This is significantly less than the observed bias and suggests that seed set alone does not explain the pattern. In addition, the proportion of seeds from dune mothers sired by nondune plants is increased when nondune pollen is given a 12-hour head start relative to a six-hour head start (χ^2_1= 6.79, P = 0.009), while the proportion of dune fathers is unaffected by a longer head start for dune pollen (χ^2_1= 0.06, P = 0.81, Fig. S9). This suggests that six hours is enough time for dune pollen to fertilize all available ovules but not enough time for nondune pollen to do the same. Therefore, nondune pollen likely has a slower or more variable fertilization rate in dune styles. This could be due to different rates of pollen germination, elongation, or acceptance.

INTRINSIC HYBRID INVIABILITY

We found that hybrid seeds germinated as well as parental seed types. Specifically, the factors maternal ecotype, paternal ecotype, and their interaction do not improve model fits (maternal: $\chi^2_1 = 1.15, P = 0.29$; paternal: $\chi^2_1 = 0.001, P = 0.97$; maternal-by-paternal: $\chi^2_1 = 0.12, P = 0.73$), nor does a factor that distinguishes hybrid and parental seed types (χ^2_1 = 0.08, P = 0.78). Accordingly, both barrier strength estimates were low ($RI_{N\to D}$ = –0.003, $RI_{D\to N}$ = 0.006) and had confidence intervals that overlapped zero (Table 2, Fig. 3).

HYBRID STERILITY

The pollen produced by hybrid plants was largely viable (93%) and comparable to the viability of pollen produced by parental types (dune = 95%, nondune = 87%, Fig. S10). There were no significant differences between pollen produced by plants crossed within and between types (χ^2_1 = 0.92, P = 0.35). Again, these estimates resulted in weak barrier strengths that are not significantly different from zero ($RI_{N\to D}$ = 0.01, $RI_{D\to N}$ = –0.03, Table 2, Fig. 3).

TOTAL REPRODUCTIVE ISOLATION

We found very strong total RI separating the ecotypes after combining multiple barriers (Table 2). The strength of RI inhibiting seed flow from the sand sheet onto the dunes was 0.999 and from the dunes to the sand sheet was 0.992, though the confidence intervals include 0 in the second case due to low survival of all seeds (Table 2). Similarly, total RI specific to pollen flow was 0.986 from sand sheet to dune and 1 from dune to sand sheet. These calculations were not particularly sensitive to the exclusion of any single barrier. The biggest drop in total barrier strength (1–0.811) was observed after excluding selection against F_1Ns from the calculation of total RI for pollen flow from the dunes to the sand sheet (not one F_1N individual survived on the sand sheet). The exclusion of any other barrier resulted in a less than 0.1 drop in total RI.

Discussion

Despite the relatively young age of the ecotypes (<10,000 years), many reproductive barriers separate dune and nondune populations of *H. petiolaris*. We found extrinsic barriers that act before and after zygote formation and include selection against immigrants, different pollinator assemblages, and selection against hybrids. In addition, we found evidence for an intrinsic barrier, postpollination assortative mating, which is likely caused by differential fertilization, possibly acting alongside ovule abortion and/or an early-acting hybrid incompatibility. Together these barriers generate strong total RI between the ecotypes.

Although seedling emergence and fecundity did not show patterns of local adaptation individually, local adaptation was apparent when a cumulative measure of fitness was analyzed. Moreover, we saw similar patterns of local adaptation in smaller reciprocal transplants conducted in 2010 and 2011 (Supporting Information Methods and Results). The number and size of seeds and their effects on seedling emergence and plant fecundity appear to be important to local adaptation in this system. Dune plants produce few large seeds that emerge better in both environments, while nondune plants produce many small seeds on the sand sheet. It is likely that differences in seed size were driven by divergent natural selection and that they contribute to selection against immigrants and hybrids with intermediate phenotypes (Rice and Hostert 1993; Hatfield and Schluter 1999). Large seeds are associated with dune adaptation in other systems (e.g., Maun 1994; Cordazzo 2002; Donovan et al. 2010), and seed manipulations in the field suggest that seed size per se is under selection in

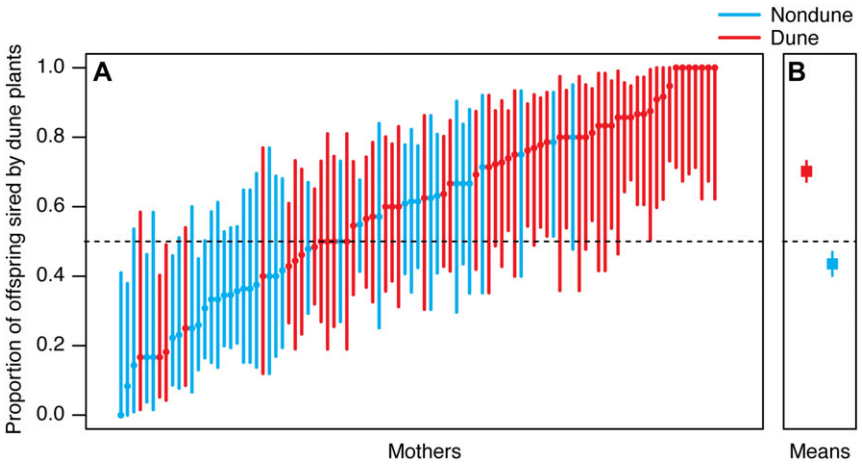

Figure 5. **Siring bias. (A) Mean siring bias for individual dune and nondune plants measured as the proportion of offspring sired by dune plants after mixed pollinations. Only plants that had at least five offspring genotyped are plotted. (B) Mean siring bias for all dune and nondune plants. Error bars are 95% confidence intervals and the dashed lines represent the expected proportion of offspring sired by dune plants (0.5).**

the sand dunes (R. L. Andrew, unpubl. data). Perhaps large seeds provide seedlings with enough resources to emerge after being buried by sand or send stabilizing taproots deep into the dunes. Alternatively or in addition, large seeds may stay closer to the surface of shifting sands, again facilitating seedling emergence.

An unexpected aspect of these results is that we observed little evidence for flowering time differences. In several plant systems (e.g., McNeily and Antonovics 1968; Hurlbert 1970; Husband and Schemske 2000; Lowery et al. 2008b; Briscoe Runquist et al. 2014), flowering time is one of the strongest barriers, favoring pollinator specialization as well as forming a barrier in its own right. There is scope for flowering time differences to evolve in the dune sunflowers, owing to the variability of flowering time, both in the field and in the greenhouse (pers. obs. and unpubl. data). Yet pollinator filtering by habitat appears to be a more important barrier to hybridization in this case. Reproductive isolation due to pollinator assemblages is probably a common consequence of moving into a new habitat but has not often been quantified (but see Kay 2006; Roccaforte et al. 2015).

The rapid evolution of postpollination assortative mating is also surprising, as this has not been found in other recently diverged ecotypes (see Husband et al. 2002 and Briscoe Runquist et al. 2014 for related examples), but it is rarely studied. Differential pollen success is thus worth investigating in other incipient species. *H. petiolaris* is self-incompatible and unlikely to be pollen limited in these populations (plants are visited by many potential pollinators and are likely limited by low nutrient soils). These conditions suggest that sexual selection could be strong (Willson and Burley 1983), and it is possible that biased siring is the result of mate choice acting through differential fertilization success or biased ovule abortion. Moreover, mate choice may have evolved in response to selection against hybrids. If so, this would represent an example of reinforcement acting early in speciation. Alternative explanations for the evolution of postpollination assortative mating are that biased siring is genetically linked or pleiotropic with other traits under selection or that the underlying genes drifted apart during an unknown period of allopatry. Future genetic analyses would allow these alternatives to be distinguished.

Overall, RI was consistently stronger from the sand sheet into the dune environment. These asymmetrical barrier strengths match population genetic estimates of gene flow that infer more gene flow onto the sand sheet than the reverse (Andrew et al., 2012, 2013). Asymmetric RI is common in studies that measure barrier strengths (e.g., Bolnick and Near 2005; Rahme et al. 2009; Sánchez Guillén et al. 2012; Ishizaki et al. 2013), although it is most often reported for postmating barriers in plants (Lowry et al. 2008a).

Also in line with previous studies (Lowry et al. 2008a), we found several strong prezygotic barriers (selection against immigrants, different pollinator assemblages, and the differential fertilization component of postpollination assortative mating). However, we also found strong postzygotic barriers caused by selection against hybrids. In fact, postzygotic barriers alone yield stronger values of total RI (seed flow: $RI_{N\to D} = 0.970$, $RI_{D\to N} = 0.906$; pollen flow: $RI_{N\to D} = 0.904$, $RI_{D\to N} = 1$) than prezygotic barriers alone (seed flow: $RI_{N\to D} = 0.935$, $RI_{D\to N} = 0.849$; pollen flow: $RI_{N\to D} = 0.749$, $RI_{D\to N} = 0.491$). Because the prezygotic barriers are not complete, the postzygotic barriers contribute meaningful reductions in gene flow between the ecotypes.

Extrinsic ecological barriers (selection against immigrants and hybrids, different pollinator assemblages) tended to be

stronger than intrinsic barriers (flowering time, postpollination assortative mating, intrinsic hybrid inviability, and sterility) in our data. As such, total isolation caused by extrinsic barriers (seed flow: $RI_{N\to D} = 0.998$, $RI_{D\to N} = 0.987$; pollen flow: $RI_{N\to D} = 0.971$, $RI_{D\to N} = 1$) was stronger than total isolation caused by intrinsic barriers (seed flow: $RI_{N\to D} = 0.261$, $RI_{D\to N} = 0.229$; pollen flow: $RI_{N\to D} = 0.447$, $RI_{D\to N} = 0.181$). This difference has been found in other systems (e.g., Ramsey et al. 2003; Kay 2006; Melo et al. 2014) and is not surprising in a system that is experiencing divergent natural selection.

Total barrier strength is strong in both directions and hinders gene flow via pollen and seeds. However, the true values of unidirectional total RI are probably closer to the pollen flow estimates because pollen flow can be an order of magnitude more common than seed flow (Petit et al. 2005), and the phase that disperses more will have a disproportionate effect on isolation. Another consideration is that our total RI calculations assume each barrier is independent of one another (Martin and Willis 2007). However, if barriers were perfectly positively correlated, some genotypes would be stopped by multiple barriers while other genotypes would be stopped by none. Even with perfect genetic correlations, however, RI is at least as high as the strongest individual barrier, which was still quite high in this study (Table 2). The nearly complete isolation resulting from these barriers explains the maintenance of separate ecotypes in the face of gene flow.

Additional caveats to our measure of total RI are that we may have missed reproductive barriers and that some of the barriers we measured required simplifying assumptions. For example, we did not account for RI mediated via pollinator constancy. Although dune and nondune flower heads look superficially similar, pollinators could discriminate between them based on unmeasured characteristics such as ultraviolet reflectance, volatile compounds, or flower head shape and size. Also, our analysis of potential pollinator assemblages makes a number of simplifying assumptions that could over- or underestimate its effect. These assumptions include that all visiting species are equally effective pollinators and that species found in both environments actually travel between the two environments. It would be useful to quantify the extent of pollinator movement between ecotypes and determine whether the primary pollinators discriminate between dune and nondune plants growing in the same population. We also did not measure ecogeographic isolation, which is a barrier caused by spatial separation as a result of genetically based ecological differences between taxa (Schemske 2000; Sobel 2014). This barrier is important in other systems (e.g., Ramsey et al. 2003; Dell'Olivo et al. 2011; Sobel 2014). However, dune *H. petiolaris* has a very small range adjacent to the much larger range of typical *H. petiolaris*, and most dune populations are within the range of potential pollen and seed dispersal from the sand sheet. Because ecogeographic isolation would not be very effective at stopping migration into the dune environment and reduced migration into the sand sheet is limited by the extent of dune environments, we focused on local processes.

One consideration when using ecotypes to study speciation is that we cannot be sure that they will complete the speciation process (Coyne and Orr 2004). It is possible that many 'incipient species' never fully speciate and instead persist at an intermediate stage of divergence. Although this scenario is possible for dune *H. petiolaris*, we believe it is relatively unlikely because the divergence is so recent and yet characterized by a surprising number of strong reproductive barriers. Furthermore, *H. neglectus*, another sand dune specialist sister to *H. petiolaris*, has completed the speciation process and exhibits many similarities to the ecotypes studied here, including divergence in seed size (Heiser 1958; Chandler et al. 1986; Raduski et al. 2010).

Conclusion

We tested dune and nondune ecotypes of *H. petiolaris* for reproductive barriers that reduce gene flow between them. This is one of very few studies investigating the evolution of RI within populations known to be separated by short time scales ($<\sim$10,000 years). We found several strong extrinsic barriers in line with local adaptation, as well as an intrinsic barrier (postpollination assortative mating). Our results highlight the importance of postzygotic barriers in addition to prezygotic barriers, even during the earliest stages of speciation. Taken together, the most striking result of these experiments is that multiple diverse reproductive barriers separate the incipient species despite recent divergence in the presence of gene flow.

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DATA ARCHIVING

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LITERATURE CITED

Andrew, R. L., and L. H. Rieseberg. 2013. Divergence is focused on few genomic regions early in speciation: incipient speciation of sunflower ecotypes. Evolution 67:2468–2482.

Andrew, R. L., K. L. Ostevik, D. P. Ebert, and L. H. Rieseberg. 2012. Adaptation with gene flow across the landscape in a dune sunflower. Mol. Ecol. 21:2078–2091.

Andrew, R. L., N. C. Kane, G. J. Baute, C. J. Grassa, and L. H. Rieseberg. 2013. Recent nonhybrid origin of sunflower ecotypes in a novel habitat. Mol. Ecol. 22:799–813.

Antonovics, J. 1968. Evolution in closely adjacent plant populations. V. Evolution of self-fertility. Heredity 23:219–238.

Aston, J. L. 1966. Evolution in closely adjacent plant populations. II. *Agrostis stolonifera* in maritime habitats. Heredity 21:649–664.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015a. lme4: linear mixed-effects models using Eigen and S4. R package ver. 1. 1-8. Available at http://CRAN.R-project.org/package=lme4.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015b. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67:1–48.

Bolnick, D. I., and T. J. Near. 2005. Tempo of hybrid inviability in centrarchid fishes (Teleostei: Centrarchidae). Evolution 59:1754–1767.

Bray, J. R., and J. T. Curtis. 1957. An ordination of the upland forest communities of southern Wisconsin. Ecol. Monogr. 27:325–349.

Briscoe Runquist, R. D., E. Chu, J. L. Iverson, J. C. Kopp, and D. A. Moeller. 2014. Rapid evolution of reproductive isolation between incipient outcrossing and selfing *Clarkia* species. Evolution 68:2885–2900.

Butlin, R. 1989. Reinforcement of premating isolation. Pp. 158–179 in Otte, D. and J. A. Endler (eds) Speciation and its consequences. Sinauer Associates, Inc., Sunderland, MA.

Chandler, J. M., and C. C. Jan. 2007. Comparison of germination techniques for wild *Helianthus* seeds. Crop Sci. 25:356–358.

Chandler J. M., C.-C. Jan, and B. H. Beard. 1986. Chromosomal differentiation among the annual *Helianthus* species. Syst. Bot. 11:354–371.

Cordazzo, C. V. 2002. Effect of seed mass on germination and growth in three dominant species in southern Brazilian coastal dunes. Braz. J. Biol. 62:427–435.

Coyne, J., and H. Orr. 2004. Speciation. Sinauer Associates, Sunderland, MA.

Dell'Olivo, A., M. E. Hoballah, T. Gübitz, and C. Kuhlemeier. 2011. Isolation barriers between *Petunia axillaris* and *Petunia integrifolia* (Solanaceae). Evolution 65:1979–1991.

Donovan, L. A., D. M. Rosenthal, M. Sanchez-Velenosi, L. H. Rieseberg, and F. Ludwig. 2010. Are hybrid species more fit than ancestral parent species in the current hybrid species habitats? J. Evol. Biol. 23:805–816.

Efron, B. 1987. Better bootstrap confidence intervals. J. Am. Stat. Assoc. 82:171–185.

Fishman, L., and J. H. Willis. 2006. A cytonuclear incompatibility causes anther sterility in Mimulus hybrids. Evolution 60:1372–1381.

Fournier, D. A., H. J. Skaug, J. Ancheta, J. Ianelli, A. Magnusson, M. N. Maunder, A. Nielsen, and J. Sibert. 2012. AD Model Builder: using automatic differentiation for statistical inference of highly parameterized complex nonlinear models. Optim. Methods Softw. 27:233–249.

Geyer, C. J. 2015. aster: aster models. R package ver. 0. 8-31. Available at http://CRAN.R-project.org/package=aster.

Geyer, C. J. 2007. Aster models for life history analysis. Biometrika. 94:415-426.

Hatfield, T., and D. Schluter. 1999. Ecological speciation in sticklebacks: environment-dependent hybrid fitness. Evolution 53:866–873.

Heiser, C. B. 1958. Three new annual sunflowers (*Helianthus*) from the southwestern United States. Rhodora 60:272–283.

Heiser, C. B., D. M. Smith, S. B. Clevenger, and W. C. Martin. 1969. The North American sunflowers (Helianthus). Seeman Printery, Durham, NC.

Hopkins, R., and M. D. Rausher. 2012. Pollinator-mediated selection on flower color allele drives reinforcement. Science 335:1090–1092.

Hurlbert, S. H. 1970. Flower number, flowering time, and reproductive isolation among ten species of *Solidago* (Compositae). Bull. Torrey Bot. Club 97:189–195.

Husband, B. C., and D. W. Schemske. 2000. Ecological mechanisms of reproductive isolation between diploid and tetraploid *Chamerion angustifolium*. J. Ecol. 88:689–701.

Husband, B. C., D. W. Schemske, T. L. Burton, and C. Goodwillie. 2002. Pollen competition as a unilateral reproductive barrier between sympatric diploid and tetraploid *Chamerion angustifolium*. Proc. R. Soc. Lond. B Biol. Sci. 269:2565–2571.

Ishizaki, S., T. Abe, and M. Ohara. 2013. Mechanisms of reproductive isolation of interspecific hybridization between *Trillium camschatcense* and *T. tschonoskii* (Melanthiaceae). Plant Species Biol. 28:204–214.

Johnson, R. B. 1968. The great sand dunes of southern Colorado. *US Geological Survey Professional Paper* 575:177–183.

Kao, J. Y., S. Lymer, S. H. Hwang, A. Sung, and S. V. Nuzhdin. 2015. Postmating reproductive barriers contribute to the incipient sexual isolation of the United States and Caribbean *Drosophila melanogaster*. Ecol. Evol. 5:3171–3182.

Kay, K. 2006. Reproductive isolation between two closely related hummingbird pollinated neotropical gingers. Evolution 60:538–552.

Kozak, G. M., A. B. Rudolph, B. L. Colon, and R. C. Fuller. 2012. Postzygotic isolation evolves before prezygotic isolation between fresh and saltwater populations of the rainwater killifish, *Lucania parva*. Int. J. Evol. Biol. 2012:1–11.

Li, N., and Y. Li. 2015. Maternal control of seed size in plants. J. Exp. Bot. 66:1087–1097.

Lowry, D. B., J. L. Modliszewski, K. M. Wright, C. A. Wu, and J. H. Willis. 2008a. The strength and genetic basis of reproductive isolating barriers in flowering plants. Philos. Trans. R. Soc. B Biol. Sci. 363:3009–3021.

Lowry, D. B., R. C. Rockwood, and J. H. Willis. 2008b. Ecological reproductive isolation of coast and inland races of *Mimulus guttatus*. Evolution 62:2196–2214.

Lynch, M., and A. G. Force. 2000. The origin of interspecific genomic incompatibility via gene duplication. Am. Nat. 156:590-605.

Martin, N. H., and J. H. Willis. 2007. Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. Evolution 61:68–82.

Matsubayashi, K. W., and H. Katakura. 2009. Contribution of multiple isolating barriers to reproductive isolation between a pair of phytophagous ladybird beetles. Evolution 63:2563–2580.

Maun, M. A. 1994. Adaptations enhancing survival and establishment of seedlings on coastal dune systems. Vegetatio 111:59–70.

McMillan, W. O., C. Jiggins, and J. Mallet. 1997. What initiates speciation in passion-vine butterflies? Proc. Natl. Acad. Sci. USA 94:8628–8633.

McNeilly, T. 1968. Evolution in closely adjacent plant populations. III. *Agrostis tenuis* on a small copper mine. Heredity 23:99–108.

McNeilly, T., and J. Antonovics. 1968. Evolution in closely adjacent plant populations. IV. Barriers to gene flow. Heredity 23:205–218.

Melo, M. C., A. Grealy, B. Brittain, G. M. Walter, and D. Ortiz-Barrientos. 2014. Strong extrinsic reproductive isolation between parapatric populations of an Australian groundsel. New Phytol. 203:323–334.

Morenno-Casasola, P. 1986. Sand movement as a factor in the distribution of plant communities in a coastal dune system. Vegetatio 65:67–76.

Mountford, M. D. 1962. An index of similarity and its application to classification problems. Pp. 43–50 *in* Murphy, P. W. (ed.) Progress in soil zoology. Butterworths, London, UK.

Nepi, M., and G. G. Franchi. 2000. Cytochemistry of mature angiosperm pollen. Pp. 45–62 *in* A. Dafni, M. Hesse, and E. Pacini, eds. Pollen and pollination. Springer-Verlag, Wien.

Nosil, P. 2007. Divergent host plant adaptation and reproductive isolation between ecotypes of *Timema cristinae* walking sticks. Am. Nat. 169:151–162.

Oksanen, J., F. G. Blanchet, R. Kindt, P. Legendre, P. R. Minchin, R. B. O'Hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, and H. Wagner. 2015. vegan: community ecology package. R package ver. 2.3-0. Available at http://CRAN.R-project.org/package=vegan.

Peters, G. 2015. userfriendlyscience: quantitative analysis made accessible. R package ver. 0. 3-0. Available at http://CRAN.R-project.org/package=userfriendlyscience.

Petit, R. J., J. Duminil, S. Fineschi, A. Hampe, D. Salvini, and G. G. Vendramin. 2005. Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. Mol. Ecol. 14:689–701.

R Core Team. 2015. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at http://www.R-project.org.

Raduski, A., L. Rieseberg, and J. Strasburg. 2010. Effective population size, gene flow, and species status in a narrow endemic sunflower, *Helianthus neglectus*, compared to its widespread sister species, *H. petiolaris*. Int. J. Mol. Sci. 11:492–506.

Rahme, J., A. Widmer, and S. Karrenberg. 2009. Pollen competition as an asymmetric reproductive barrier between two closely related *Silene* species. J. Evol. Biol. 22:1937–1943.

Ramsey, J., H. Bradshaw Jr., and D. Schemske. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). Evolution 57:1520–1534.

Renaut, S., C. J. Grassa, S. Yeaman, B. T. Moyers, Z. Lai, N. C. Kane, J. E. Bowers, J. M. Burke, and L. H. Rieseberg. 2013. Genomic islands of divergence are not affected by geography of speciation in sunflowers. Nat. Commun. 4:1–8.

Rice, W. R., and E. E. Hostert. 1993. Laboratory experiments on speciation: what have we learned in 40 years? Evolution 47:1637–1653.

Rieseberg, L., A. Desrochers, and S. Youn. 1995. Interspecific pollen competition as a reproductive barrier between sympatric species of *Helianthus* (Asteraceae). Am. J. Bot. 82:515–519.

Roccaforte, K., S. E. Russo, and D. Pilson. 2015. Hybridization and reproductive isolation between diploid *Erythronium mesochoreum* and its tetraploid congener *E. albidum* (Liliaceae). Evolution 69:1375–1389.

Rundle, H. D., and D. Schluter. 1998. Reinforcement of stickleback mate preferences: sympatry breeds contempt. Evolution 52:200–208.

Rundle, H. D., L. Nagel, J. W. Boughman, and D. Schluter. 2000. Natural selection and parallel speciation in sympatric sticklebacks. Science 287:306–308.

Sambatti, J. B. M., and K. J. Rice. 2006. Local adaptation, patterns of selection, and gene flow in the Californian serpentine sunflower (*Helianthus exilis*). Evolution 60:696–710.

Sambatti, J. B. M., J. L. Strasburg, D. Ortiz-Barrientos, E. J. Baack, and L. H. Rieseberg. 2012. Reconciling extremely strong barriers with high levels of gene exchange in annual sunflowers. Evolution 66:1459–1473.

Sánchez-Guillén, R. A., M. Wellenreuther, and A. Cordero Rivera. 2012. Strong asymmetry in the relative strengths of prezygotic and postzygotic barriers between two damselfly sister species. Evolution 66:690–707.

Schemske, D. W. 2000. Understanding the origin of species. Evolution 54:1069–1073.

Schluter, D. 2009. Evidence for ecological speciation and its alternative. Science 323:737–741.

Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9:671–675.

Scopece, G., A. Croce, C. Lexer, and S. Cozzolino. 2013. Components of reproductive isolation between *Orchis mascula* and *Orchis pauciflora*. Evolution 67:2083–2093.

Skaug, H., D. Fournier, B. Bolker, A. Magnusson, and A. Nielsen. 2014. Generalized Linear Mixed Models using AD Model Builder. R package ver. 0.8.0.

Sobel, J. M. 2014. Ecogeographic isolation and speciation in the genus *Mimulus*. Am. Nat. 184:565–579.

Sobel, J. M., and G. F. Chen. 2014. Unification of methods for estimating the strength of reproductive isolation. Evolution 68:1511–1522.

Sobel, J. M., and M. A. Streisfeld. 2015. Strong premating reproductive isolation drives incipient speciation in *Mimulus aurantiacus*. Evolution 69:447–461.

Therneau, T. 2015. A package for survival analysis in S. R package ver. 2.38. Available at http://CRAN.R-project.org/package=survival.

Therneau, T. M., and P. M. Grambsch. 2000. Modeling survival data: extending the Cox model. Springer, New York.

Westoby, M., E. Jurado, and M. Leishman. 1992. Comparative evolutionary ecology of seed size. Trends Ecol. Evol. 7:368–372.

Wickham, H. 2011. The split-apply-combine strategy for data analysis. J. Stat. Softw. 40:1–29. Available at http://www.jstatsoft.org/v40/i01.

Willson, M. F. and N. Burley. 1983. Mate choice in plants: tactics, mechanisms, and consequences. Princeton University Press, Princeton, New Jersey.

Yatabe, Y., N. C. Kane, C. Scotti-Saintagne, and L. H. Rieseberg. 2007. Rampant gene exchange across a strong reproductive barrier between the annual sunflowers, *Helianthus annuus* and *H. petiolaris*. Genetics 175:1883–1893.

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Supporting Information

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

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