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HYBRID INCOMPATIBILITY IS ACQUIRED FASTER IN ANNUAL THAN IN PERENNIAL SPECIES OF SUNFLOWER AND TARWEED

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Hybrid sterility is an important species barrier, especially in plants where hybrids can often form between divergent taxa. Here we explore how life history affects the acquisition of hybrid sterility in two groups in the sunflower family. We analyzed genetic distance and F1 pollen sterility for interspecific crosses in annual and perennial groups. We find that reproductive isolation is acquired in a steady manner and that annual species acquire hybrid sterility barriers faster than perennial species. Potential causes of the observed sterility pattern are discussed.

KEY WORDS: **Evolutionary rate, karyotypic changes, life history, plants, speciation.**

Speciation is characterized by the evolution of reproductive isolation. This can come in many forms including prezygotic barriers such as reproductive timing and gametic incompatibility or postzygotic barriers such as hybrid viability or sterility (Coyne and Orr 2004; Rieseberg and Willis 2007). The speed with which these barriers arise and the impact of life-history variation on their evolution remain poorly understood (Edmands 2002). In plants it is common for well-recognized species to be able to interbreed and produce hybrids of varying levels of fertility (Levin 1979). These intermediates can be used to study how intrinsic reproductive isolation evolves.

That different plant species can interbreed is not a new discovery. This has been recognized since the 18th century and during the mid-20th century hybridization between taxa was widely employed to estimate phylogenetic relationships (Turesson 1929; Zirkle 1935; Levin 1979; Edmands 2002). Species with hybrids that had greater F1 viability or fertility were judged to be more closely related. This rich dataset can be combined with modern sequencing efforts, which more precisely estimate divergence between species, to explicitly examine the relationship between genetic divergence and the strength of reproductive isolation.

In animals, it is widely accepted that reproductive isolation evolves in a relaxed clock-like manner. This has been shown in a variety of taxa including fish, birds, frogs, flies, and butterflies (Sasa et al. 1998; Presgraves 2002; Price and Bouvier 2002; Lijtmaer et al. 2003; Russell 2003; Bolnick and Near 2005). In plants the relationship is less clear; a loosely clock-like relationship was found in *Silene* and *Coreopsis* but not in *Glycine, Streptanthus*, and *Frageria* (Moyle et al. 2004; Nosrati et al. 2011). This may reflect inherent differences in the genetic architecture of reproductive isolation. If many genes of small effect cause isolation, then a clear relationship will occur. Alternatively, if few genes (or chromosomal rearrangements) of large effect cause isolation, then stochastic variation among lineages may obscure any relationship (Edmands 2002).

Several biological factors have been shown to affect the rate of reproductive barrier evolution, including the degree of sympatry between species, the presence of sex chromosomes, and the extent of ecological divergence (Coyne and Orr 1989; Nosil

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and Crespi 2006; Yukilevich 2012). Life history, annuals versus perennials, is associated with the evolution of reproductive isolation in the plant genus *Coreopsis* (family Asteraceae): annuals were found to accumulate hybrid incompatibilities more quickly than perennials (Archibald et al. 2005). However, this pattern has not been tested beyond this single genus. To determine whether this is a more general phenomenon, we analyzed the relationship between life history and the strength of hybrid sterility barriers in two independent clades containing both extensive crossing data and life-history variation, the genus *Helianthus* and subtribe Madiinae.

Helianthus (family Asteraceae) comprises 52 species, all native to North America. One of these is the common sunflower, *H. annuus*, which includes both the cultivated sunflower—an important crop—and its wild progenitor. The genus has been studied extensively for both agricultural and evolutionary purposes, resulting in a rich literature on chromosomal evolution and speciation (Rieseberg et al. 1995; Jan 1997; Archibald et al. 2005; Lai et al. 2005). Subtribe Madiinae (family Asteraceae) contains 24 genera and 121 species. This includes the tarweeds of California and silverswords of the Hawaiian Islands. The silverswords underwent a rapid radiation into many morphological forms but retained the ability to hybridize (Carr and Kyhos 1986). In both cases, older crossability data can be combined with more recent sequence data.

Here we have compiled pollen sterility and sequence data from artificial crosses between *Helianthus* and Madiinae species. We use these data to ask two questions: (1) does reproductive isolation accrue in a clocklike manner? and (2) do annuals gain hybrid sterility faster than perennials? Additionally, we discuss possible causes of the differences in the rate of sterility evolution.

Methods

DATA COLLECTION

Information on pollen sterility between *Helianthus* and Madiinae species was taken from the literature (Appendix S1). *Helianthus* data included only crosses between sunflower species, whereas the Madiinae data included crosses between multiple genera of tarweeds. Artificial and natural hybrids were distinguished and only artificial crosses were used in our analysis. Direction of crosses was not distinguished as this information was not available for all crosses.

Ten Madiinae crosses involved second-generation hybrids, for example, *Dubautia knudsenii* × *D. laxa* crossed to *D. latifolia*. In these cases, the genetic distance used was the mean of the genetic distance from the first two species to the third species. These crosses were included in the phylogenetically corrected dataset only when the first two parental species were more closely related to each than to the third species, that is, when there was an unambiguous internal node. For the analysis of life history, these crosses were included because in each case all three parents were perennial, making assignment unambiguous. Life history was recorded as annual or perennial for each species. Thus crosses were annual–annual, perennial–perennial, or annual–perennial.

Genetic distance was calculated from sequences of the external transcribed spacer (ETS) and the internal transcribed spacer (ITS) of 18S-26S nuclear ribosomal DNA for *Helianthus* and Madiinae, respectively. All sequences were obtained from Genbank (Appendix S2). Sequences were aligned using ClustalW (Larkin et al. 2007) and pairwise distance was calculated using MEGA5 (Tamura et al. 2011). Model test was used to determine the correct model of sequence evolution and only sites with $\geq$95% coverage were used (Posada and Crandall 1998).

PHYLOGENETIC INDEPENDENCE

Due to the nature of our dataset, the information provided by each individual cross was not phylogenetically independent. To alleviate this issue, we created a "phylogenetically corrected" dataset (Coyne and Orr 1997). This collapsed all pairwise comparisons across a single internal node into a single datapoint. Although this method does not provide complete phylogenetic independence, it is commonly used and ensures that any two datapoints do not share more than 50% of their phylogenetic history (Price and Bouvier 2002; Moyle et al. 2004; Larkin et al. 2007; Malone and Fontenot 2008).

Phylogenies for both datasets were taken from previously published work. For the *Helianthus* dataset, the phylogeny was based on the same ETS sequences used to estimate genetic distance (Timme et al. 2007). For the Madiinae, no single published phylogeny covered our entire dataset of species so a consensus of multiple phylogenies was used. These phylogenies are based on ITS sequences (*Layia*, Baldwin 2003; *Argyroxiphium*, *Dubautia*, *Wilkesia*, Baldwin and Sanderson 1998), both ETS and ITS (*Calycadenia*, Baldwin and Markos 1998; *Deinandra*, Baldwin 2007), ETS, ITS, and the *trnK* intron of chloroplast DNA (Madiinae, Baldwin 2003). Phylogenetic trees with nodes labeled are presented in Figures S1 and S2.

To assess the effect of life history on the evolution of hybrid sterility, the dataset was first divided according to life cycle and then phylogenetically collapsed into independent nodes. The data were then brought back together into a single dataset with independent datapoints of either type. Thus a single node on a tree may be represented in two separate categories, for example, contain both an annual–annual and perennial–perennial comparison. The shared evolutionary history for these datapoints may obscure any differences in rate, but overall makes our test conservative in its conclusions.

Our method of assessing the effect of life history is simpler than the method used by Archibald et al. (2005), who assessed reproductive isolation in relation to annual or perennial *branch length*, but does not suffer from phylogenetic independence issues. Our test is likely less powerful but more conservative and does not rely upon the ability of the relatively short markers used to accurately reconstruct the phylogenetic relationships among the focal species.

STATISTICAL ANALYSIS

We used genetic distance as a proxy for divergence times in our analysis. This relationship may be complicated by uneven rates of evolution or ongoing gene flow between species (but see discussion). As both pollen sterility and genetic distance were not normally distributed, both variables were arcsine transformed. We compared genetic distance and pollen sterility between Madiinae crosses that were first- and second-generation hybrids (hybrid–hybrids) using a Kruskal–Wallis test (Kruskal and Wallis 1952). Transformed data were used to test for a correlation between pollen sterility and genetic distance using a nonparametric Spearman rank correlation to account for any residual nonnormality.

To determine if life history affects the rate of reproductive isolation acquisition, we used an analysis of variance (ANOVA). We fit a linear model testing the effect of genetic distance, life history, and their interaction on pollen sterility using the statistical programs in R (Ihaka and Gentleman 1996).

TESTING EVOLUTIONARY RATE

Evolutionary rate was measured by comparing genetic distance between monophyletic groups of perennial or annual species with an outgroup that was equally related to all groups. Groups are indicated in Figures S1 and S2. Genetic distance was measured with MEGA5 using Jukes–Cantor model with gamma parameter = 1 and complete deletion for missing positions.

Results

DATASET

In *Helianthus* and Madiinae, we compiled data for 114 and 87 crosses representing 43 and 47 species, respectively. This included both within genera and between genera crosses as well as crosses where one or both of the parents were themselves an F1 hybrid. These second generation hybrids were not different from the rest of the dataset in genetic distance or pollen sterility (df = 1, $P = 0.594$; $P = 0.739$).

After collapsing the data to only phylogenetically independent nodes, 20 and 30 datapoints remained (shown in Figs. S1 and S2). The low number of independent nodes in the *Helianthus* dataset is largely because of two reasons. First, the genus is divided into perennial and annual clades so all crosses between these clades (43 separate hybrids) are reduced to three nodes. Second, the perennial species are poorly resolved and many are not monophyletic. We were conservative in our use of these data so several species' relationships were reduced to single polytomies.

THE RELATIONSHIP BETWEEN POLLEN STERILITY AND GENETIC DISTANCE

There was a clear positive relationship between pollen sterility and genetic distance before phylogenetic correction for both Madiinae (rho = 0.50, $P < 10^{-6}$) and *Helianthus* (rho = 0.44, $P < 10^{-6}$) datasets. In the phylogenetically independent datasets, this relationship is maintained for Madiinae (rho = 0.61, $P < 0.001$) but for *Helianthus* it is no longer significant (rho = 0.39, $P = 0.09$; Table 1).

LIFE-HISTORY DIFFERENCES

Life history had a large effect in both datasets. Annual–annual crosses were much more strongly isolated than perennial–perennial crosses in terms of hybrid pollen viability (Fig. 1). In both cases, when accounting for genetic distance, life history explained a significant portion of the variance in sterility (Table 2).

COMPARISONS OF RATES OF SEQUENCE EVOLUTION

For *Helianthus* data, perennial groups had mean genetic distances of 0.054 and 0.057, and the annuals had a mean distance of 0.064. For Madiinae, two paired perennial and annual clades had mean genetic distances of 0.098 versus 0.104 and 0.075 versus 0.084, respectively. In both cases, annual clades exhibited greater genetic distance.

Discussion

HYBRID STERILITY INCREASES WITH GENETIC DISTANCE

It is intuitively obvious that reproductive isolation is correlated with genetic distance. Before populations diverge they should have little or no reproductive isolation and no genetic distance. Conversely, distantly related species have total reproductive isolation and high genetic distance. Positive correlation between genetic distance and sterility has been found repeatedly in animals, including Drosophila (Coyne and Orr 1997), frogs (Sasa et al. 1998), toads (Malone and Fontenot 2008), fish (Russell 2003), birds (Price and Bouvier 2002), and butterflies (Presgraves 2002). Despite this, evidence for this pattern has been relatively scarce in plants; it was found in *Silene* and *Coreopsis* but missing in *Glycine*, *Streptanthus*, and *Frageria* (Moyle et al. 2004; Archibald et al. 2005; Nosrati et al. 2011). Here we show strong evidence for this relationship in both *Helianthus* (sunflowers) and Madiinae (tarweeds).

Table 1. Correlations between genetic distance and pollen viability for all comparisons and for the phylogenetically corrected dataset.

	Number of species	N Crosses$_{original}$	Spearman's rho$_{original}$		N Crosses$_{corrected}$	Spearman's rho$_{corrected}$	
Helianthus	43	114	0.44	$\mathbf{P < 10^{-6}}$	20	0.39	$P = 0.09$
Madiinae	47	87	0.50	$\mathbf{P < 10^{-6}}$	30	0.61	$\mathbf{P < 0.001}$

Significant *P* values below 0.05 are highlighted in bold.

The positive correlation between reproductive isolation and genetic distance suggests that reproductive isolation is acquired in a relaxed clock-like manner. This occurs despite evidence that chromosomal rearrangements play a significant role in generating sterility (see below).

LIFE HISTORY

Our analysis clearly shows that annual species develop F1 hybrid sterility at a faster rate than perennials. Annual–annual crosses have mean pollen sterility of 90% (*Helianthus*) and 93% (Madiinae) versus 41% and 55% for perennial–perennial crosses. In fact, there are no annual–annual crosses with less than 57% sterility despite the inclusion of crosses between sister species.

It is interesting to note that although hybrids between perennial sunflowers are highly fertile, there seems to be a strong barrier to hybrid seed production (Heiser et al. 1969). Artificial crosses between perennial species require huge amounts of effort to obtain

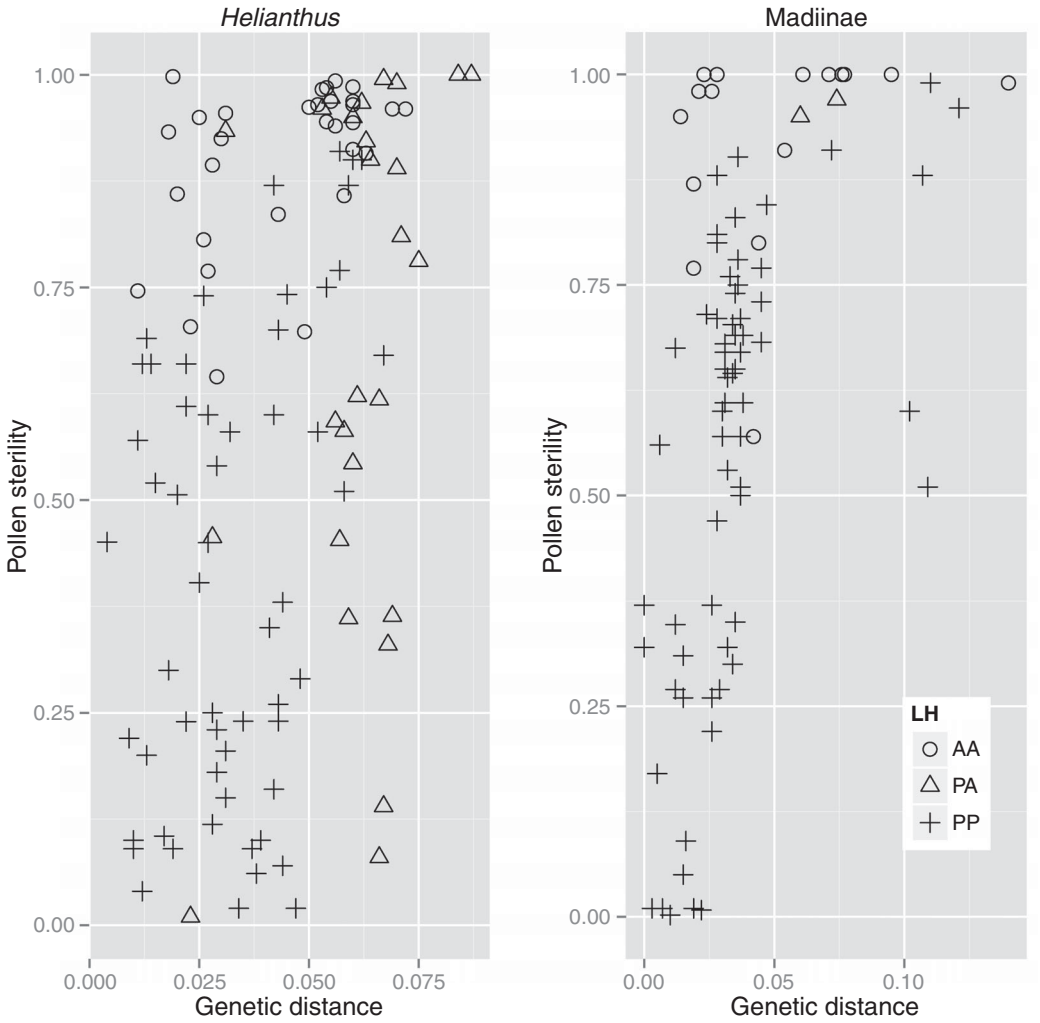

Figure 1. Pollen sterility and genetic distance for *Helianthus* and Madiinae datasets. Individual points are not phylogenetically corrected and are coded by life-history combination. A is annual, P is perennial. Genetic distance was measured using ITS (Madiinae) or ETS (*Helianthus*).

Table 2. Results of analysis of variance for all variables tested using phylogenetically corrected datasets. Genetic distance is arcsine transformed in all cases.

	Variable	df	Sum sq.	Mean sq.	*F*-value	*P*
Helianthus	Genetic distance	1	0.7142	0.7142	14.0678	**0.001259**
	Life history	2	0.90428	0.45214	8.9059	**0.001714**
	Genetic distance × life history	2	0.06629	0.03315	0.6529	0.531289
	Residuals	20	1.01537	0.05077		
Madiinae	Genetic distance	1	2.01713	2.01713	30.471	$\mathbf{9.77 \times 10^{-6}}$
	Life history	2	1.96053	0.98026	14.808	$\mathbf{5.73 \times 10^{-5}}$
	Genetic distance × life history	1	0.20736	0.20736	3.1324	0.08895
	Residuals	25	1.65496	0.0662		

Significant *P* values below 0.05 are highlighted in bold.

a few viable seeds; indeed, modern crosses involving perennial sunflowers often use embryo rescue (Kräuter et al. 1991).

EVOLUTIONARY RATE

Our study uses genetic distance as a proxy for divergence time. This is not a perfect measure as rates of sequence evolution vary between lineages and, most relevantly, between life-history strategies (Gaut et al. 2011). Several studies have shown that molecular evolutionary rates are faster in annuals than in perennials (Andreasen and Baldwin 2001; Kay et al. 2006; Soria-Hernanz et al. 2008); when taken into account with our results, this actually accentuates the pattern we find. If annuals evolve unusually fast in terms of nucleotide sequence, then annual–annual comparisons have lower divergence *times* and are *younger* than expected based on sequence divergence. Conversely, perennial–perennial pairs are *older* than what our sequence divergence suggests. Consider a scenario where there was no effect of life history and reproductive isolation evolved in a rate purely proportional to divergence time. Two pairs of species, one annual–annual and one perennial–perennial, that have been diverging for equal amounts of time would have equal reproductive isolation, but the annual–annual pair would have higher sequence divergence and, consequently, according to our measure, a slower rate of reproductive isolation gain. This is the opposite of the pattern we observe in the data; therefore differences in the rate of sequence evolution are not driving the patterns we see.

To confirm the differences in sequence divergence rate, we examined evolutionary rate in our dataset by comparing mean genetic distance between annual and perennial groups to outgroups (Figs. S1 and S2). In all cases annual clades had greater genetic distance, suggesting faster sequence evolution. The variation between Madiinae pairs may represent long-term differences in rates of sequence divergence as these comparisons are between different genera. In each case, annual groups evolved faster in terms of nucleotide sequence than perennial groups. Thus, the more rapid evolution of hybrid sterility barriers in annuals does not appear to be a consequence of misestimating divergence times. Rather, differences in rates of sequence evolution appear to be causing the trend to be underestimated.

It is also possible that the low levels of hybrid sterility found between perennial species may permit significant interspecific gene flow, thereby reducing genetic divergence. However, this seems unlikely for perennial sunflowers, which appear to be reproductively isolated by strong prezygotic reproductive barriers. Also, this scenario does not explain why annuals developed high levels of reproductive isolation and perennials did not.

CAUSES OF STERILITY

Hybrid sterility can be caused by epistatic interactions (also known as Dobzhansky–Muller [DM] incompatibilities) or chromosomal rearrangements. DM incompatibilities are negative epistatic interactions in hybrids originating from genes that evolved independently in the parental species. Chromosomal rearrangements, on the other hand, cause sterility through the production of chromosomally unbalanced gametes hybrids (Coyne and Orr 2004). Although both cause sterility, there are distinct effects. DM incompatibilities typically are recessive and may therefore be masked in the F1 and only appear in the F2 generation, leading to increased sterility in second-generation hybrids. Chromosomal rearrangements, on the other hand, are underdominant and would thus have the greatest effect in the F1, where all polymorphic loci are heterozygous. In the F2 generation, heterozygosity is reduced and so sterility from chromosomal rearrangements will stay constant or be reduced. Additionally, in the absence of sex chromosomes, chromosomal rearrangements are symmetrical in their effect on sterility; it does not matter which species is the mother. DM incompatibilities can be bidirectional, like chromosomal rearrangements, or unidirectional and cause asymmetric sterility (Turelli and Moyle 2007). Lastly, artificial genome doubling using colchicine creates hybrids with perfectly paired chromosomes, alleviating the effect of chromosomal rearrangements but not DM incompatibilities (Stebbins 1958).

Based on these features, we have several reasons to believe that in these systems hybrid sterility is largely caused by

chromosomal changes. Pollen sterility has been mapped to chromosomal rearrangements in *Helianthus* (Quillet et al. 1995; Lai et al. 2005), although epistatic interactions between sterility QTLs suggest DM incompatibilities contribute as well. Furthermore, among F1 *Helianthus* hybrids, pollen sterility was correlated with number of chromosomal translocations, although insignificantly (Chandler et al. 1986; Levin 2002). Similarly, in Hawaiian silverswords (subtribe Madiinae) the number of translocations between parental species is strongly correlated with pollen sterility in hybrids (Carr and Kyhos 1981, 1986; Levin 2002). Chromosomal rearrangements have been extensively noted in both studied groups (Carr and Kyhos 1986; Chandler et al. 1986).

Asymmetry of sterility and the relative sterility of F1 versus F2 generations are not commonly reported or tested in our dataset so we cannot formally test them, but we examine the available data here. Cross-sterility symmetry was not reported for Madiinae crosses, but for *Helianthus* crosses are generally found to be symmetrical (Long 1955; Lai et al. 2005), suggesting little contribution from unidirectional DM incompatibilities. In hybrids between the annual sunflowers *H. anunus* and *H. petiolaris*, pollen viability significantly increases from the F1 generation ($5.6 \pm 2.2\%$, $n = 20$) to the F2 ($31.6 \pm 12.4\%$, $n = 20$) (*t*-test, $P < 0.0001$; Rieseberg 2000). Contrary to this, in hybrids between the perennial sunflowers *H. decapetalus* and *H. laevigatus*, viability decreased from the F1 (80%) to the F2 (66%) generation (Heiser and Smith 1964). Lastly, colchicine-induced chromosome doubling, which helps alleviates chromosomal mispairing, has increased pollen fertility in several sunflower hybrids (Heiser and Smith 1964; Jan and Chandler 1989).

We believe this evidence is consistent with the idea that chromosome rearrangements are important in the hybrid sterility we measured, although almost certainly not the only cause. If we accept the importance of rearrangements, why are these rearrangements occurring more frequently or being fixed more often in annuals than perennials? More specifically, we would suggest that there are more karyotypic changes per nucleotide substitution in annuals than perennials. This could be because chromosomal rearrangements occur more frequently or because demographic or selective factors cause them to fix at a greater rate. There are biological features that promote both of these options.

It is generally believed that chromosomal rearrangements primarily occur during meiosis mediated by the double strand breaks used in homologous recombination (Shaffer and Lupski 2000). By regenerating from seed every year, annuals may undergo more frequent meiosis events than perennials and accrue more chromosomal rearrangements as a consequence.

The increased chromosomal evolution may also be due to a difference in fixation rather than mutation rate. When faster sterility acquisition in annuals was first described by Stebbins (1958), he suggested that intense population fluctuations allow annuals to fix underdominant genic or chromosomal changes faster than perennials, which have more stable population sizes (Stebbins 1958). This intuitive explanation was later formalized by mathematical models demonstrating that chromosomal rearrangements could only be established in very small or inbred populations (Walsh 1982). Counter to this, in our dataset annual sunflowers, which have extremely high rates of chromosomal evolution (Burke et al. 2004), also have very high effective population size (Strasburg et al. 2011) indicating few species-wide bottlenecks. Within Madiinae, a majority of perennial crosses, which have relatively low sterility, involve silverswords, a group that speciated within the Hawaiian Islands and underwent repeated population bottlenecks (Witter and Carr 1988). Grant (1981) later suggested that higher levels of selfing in annuals also contributed to higher rates of karyotypic evolution (Grant 1981). Although selfing annuals may have high rates of chromosomal evolution, this does not explain the results reported here. In our datasets all species, including the annuals, are self-incompatible (with the exception of *H. agrestis,* which has 100% pollen sterility in both available crosses). Thus, differences in the fixation rate of karyoptypic changes due to variation in effective population size or mating system cannot account for the pattern in our dataset.

Conclusions

A major goal of studies of speciation is to identify and order the specific biological features affecting the acquisition of reproductive isolation. In our study, the effects of variation in plant growth form on the accumulation of hybrid incompatibilities were examined. We found that annuals evolve a hybrid sterility barrier much faster than perennials, and hypothesize that it is due to more rapid chromosomal evolution in annuals. The occurrence of chromosomal changes may differ simply because of differences in the ratio of meiotic to mitotic events in the different growth forms, but this does not explain how these potentially strongly underdominant rearrangements are fixing in annual species with large population sizes. Future research could test these hypotheses by (1) examining the rate of karyotypic evolution for annuals and perennials in a phylogenetic context; (2) use chromosome doubling to test the contribution of chromosome rearrangements to sterility in hybrids of both life-history strategies; and (3) examine the fitness consequences of rearrangements in the context of different life-history strategies.

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Supporting Information

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

Figure S1. Phylogeny of *Helianthus* used for creating the phylogenetically corrected dataset.
Figure S2. Phylogeny of Madiinae used for creating the phylogenetically corrected dataset.
Appendix S1. Raw data used for analysis.
Appendix S2. Accession numbers for molecular sequence used.
Supporting Information 1. References used for crossability data, see Appendix S1.