American Journal of Botany
Celebrating 100 years 1914–2014

American Journal of Botany 101(1): 180–189. 2014.

MATING BETWEEN *ECHINACEA ANGUSTIFOLIA* (ASTERACEAE) INDIVIDUALS INCREASES WITH THEIR FLOWERING SYNCHRONY AND SPATIAL PROXIMITY[1]

JENNIFER L. ISON[2,3,5,6], STUART WAGENIUS[3], DIEDRE REITZ[4], AND MARY V. ASHLEY[2]

[2]Department of Biological Sciences, University of Illinois at Chicago, 845 West Taylor Street, Chicago, Illinois 60607 USA;
[3]Plant Conservation and Biology, Chicago Botanic Garden, 1000 Lake Cook Road, Glencoe, Illinois 60022 USA;
and [4]Department of Biology, Carleton College, One North College Street, Northfield, Minnesota 55057 USA

- *Premise of the study:* Although spatial distance is considered the primary factor in determining plant mating patterns, flowering time and synchrony are also likely to be important.
- *Methods:* We quantified the relationships of both distance and flowering phenology to the probability of mating between individual plants. In an experimental plot, we followed daily flowering phenology in *Echinacea angustifolia,* a self-incompatible perennial pollinated by solitary bees. We assigned paternity to 832 of 927 seedlings from 37 maternal plants using 11 microsatellite loci. Potential pollen donors included the experiment plot's 202 flowering plants and a nearby plot's 19 flowering plants. For each maternal plant sampled, we examined the pollen pool by quantifying correlated paternity and the effective number of pollen donors.
- *Key results:* Significantly more pollinations occurred between neighboring and synchronous plants than expected under random mating, with distance being more important than flowering synchrony. The distance pollen moved varied over the course of the season, with late flowering plants mating with more distant plants compared to early or peak flowering plants. All maternal plants had a diverse set of mates (mean number of effective pollen donors = 23.7), and the composition of the pollen pools overlapped little between maternal plants.
- *Conclusion:* Both distance and flowering synchrony influenced pollination patterns in *E. angustifolia*. Our results suggest that pollen movement between incompatible mates and flowering asynchrony could be contributing to the reduced seed set observed in small *E. angustifolia* remnants. However, we also found that individual plants receive pollen from a diverse group of pollen donors.

Key words: flowering phenology; mating patterns; microsatellites; paternity analysis; phenological assortative mating; spatial isolation; tallgrass prairie.

For outcrossing plants to successfully reproduce, pollen must move from one plant to another. The movement of pollen determines both the abundance and diversity of pollen that a plant receives. Gene dispersal via pollen is predicted to be leptokurtic, with high dispersal close to the parental plant (Levin and Kerster, 1974). Flowering synchrony is also required for successful pollen movement. If the recipient plant's styles are not receptive when pollen moves, pollination cannot occur. Over generations, isolation by distance can lead to spatial genetic structure (Wright, 1943) and it is hypothesized that isolation by time can lead to temporal genetic structure (Hendry and Day, 2005). Similar to spatial genetic structure, temporal genetic structure is the nonrandom temporal distribution of alleles. The relative importance of spatial distance and flowering synchrony on the probability of mating between plants is largely unknown.

Pollen movement distances have been indirectly estimated from pollinator observation (e.g., Schmitt, 1983; Fenster, 1991) and the spatial genetic structure of plant populations (e.g., Rousset, 1997; Hardy and Vekemans, 1999). Highly variable molecular markers such as microsatellites, allow direct measures of gene dispersal through paternity analysis of progeny (reviewed in Ashley, 2010) or even individual pollen grains (Hasegawa et al., 2009). These studies revealed that pollen dispersal kernels are leptokurtic, but fat-tailed, and that long distance pollination events are common (e.g., Nason and Hamrick, 1997; Dow and Ashley, 1998; White et al., 2002; Gaiotto et al., 2003; Kramer et al., 2008; Slavov et al., 2009).

Flowering asynchrony between plants may cause temporal isolation just as spatial distance between plants causes spatial isolation. Asynchronous flowering may result in phenological assortative mating, with early flowering plants mating more frequently with other early flowering plants than late flowering plants (Wagener, 1976; Kirkpatrick, 2000; Fox, 2003; Weis and Kossler, 2004). Although abiotic and biotic factors affect flowering

[1]Manuscript received 14 February 2013; revision accepted 14 October 2013.

We would like to thank G. Keifer and the 2005 *Echinacea* Project field crew for their assistance with collecting flowering data and seeds; J. Fant, D. Feng, K. Feldheim, J. Friedman, and L. Rosenthal for their molecular genetic work assistance; E. Austen and G. Legault for the R code; and D. Zaya for assistance with the N_{ep} calculations. Funding was provided by the Millennium Seed Bank (RBG-Kew) research grant to JLI. The National Science Foundation (NSF) supported this work through grants 0083468, 0545072, 0521245, and 0544970. Fellowship support for J.L. Ison was provided by the NSF IGERT program (grant 059245) and the University of Illinois at Chicago graduate program. This work was completed in partial fulfillment of the requirements for the doctoral degree (to J.L. Ison) from the Graduate College, University of Illinois at Chicago.

[5]Author for correspondence (e-mail: ison.jennifer@gmail.com)

[6]Present address: Biology Department, Wabash College, 301 West Wabash Ave., Crawfordsville, Indiana 47933 USA.

doi:10.3732/ajb.1300065

phenology (reviewed in Rathcke and Lacey, 1985; Elzinga et al., 2007), there is often a strong genetic component of flowering time (e.g., Best and McIntyre, 1972; O'Neil, 1997; Franks et al., 2007). To the extent that flowering time is heritable, assortative mating over generations could lead to temporal genetic structure and result in temporal genetic differentiation, especially for genes correlated with flowering time (Hendry and Day, 2005).

Few studies have quantified temporal genetic structure. Studies that applied traditional population differentiation methods found no evidence for it (Stanton et al., 1997; Okayama et al., 2003; Hirao and Kudo, 2008). However, population differentiation methods, such as F_{ST}, may not be sensitive enough to detect a difference between flowering plants categorized as early or late, because there is undoubtedly substantial gene flow between those plants (Hendry and Day, 2005). A different approach, which examined continuous measures of relatedness between individuals with similar flowering times, found evidence of temporal genetic structure in a tropical tree species (Dainou et al., 2012). More studies using a variety of approaches are needed before we know if temporal genetic structure is common in plant populations.

A handful of studies used molecular markers to examine the effects of flowering phenology on pollination. Flowering phenology of the tropical tree, *Pachira quinata,* in fragmented and continuous forest populations influenced the number of sires per maternal plant (Fuchs et al., 2003). Trees from isolated patches that flowered before or after peak flowering had progeny with higher mean relatedness. A study of the woodland herbaceous plant, *Primula sieboldi,* used paternity analysis to demonstrate that phenology influences pollination distances (Kitamoto et al., 2006). On average, plants flowering either before or after peak were pollinated by plants that were 10 m farther away. Pollination distance also increased in individuals flowering off-peak in the tree, *Sorbus torminalis* (Oddou-Muratorio et al., 2006). These studies examined how seasonal flowering timing changes pollination distances, but did not quantify how flowering synchrony between plants influences the probability of mating. Another study demonstrated the importance of both spatial and temporal pollen movement for the genetic structure of plant populations. Gérard et al. (2006) found that phenological assortative mating and short-distance pollen movement counteract the effects of long distance pollen dispersal and help maintain a hybrid zone between two ash species.

In self-incompatible species, spatial genetic structure likely influences mating patterns. In a fragmented population of *Echinacea angustifolia*, plants in small remnants were often surrounded by incompatible plants (Wagenius et al., 2007). In such cases, pollen movement between many neighboring plants will not result in fertilization, and mating patterns may differ strikingly from patterns of pollen movement. In addition, results may differ among populations, because spatial genetic structure can vary by population size and fragmentation levels (e.g., Yamagishi et al., 2007; Ison, 2010; Barluenga et al., 2011). Studying mating patterns in populations without spatial genetic structure, such as an experimental plot, allows one to test the effects of distance and phenology without confounding effects of incompatibility.

To assess how a plant's location and flowering phenology influences mating patterns, we used microsatellites to conduct paternity analysis on offspring from 37 maternal plants in an experimental plot with 204 flowering *E. angustifolia* plants (Wagenius et al., 2010). In this plot, seedling placement was randomized to eliminate genetic structure and potential confounding environmental factors. Through paternity analysis, we (1) investigated how mating patterns are influenced by spatial isolation, flowering phenology, and their interaction; and (2) quantified how spatial isolation and flowering phenology influence the diversity of pollen a plant receives.

MATERIALS AND METHODS

Study species—*Echinacea angustifolia* DC, the narrow-leaved purple coneflower, is widely distributed, ranging from Texas to Canada. It is long-lived, with an estimated generation time of 17–44 years in Kansas (Hurlburt, 1999), and comparable longevity in Minnesota (Wagenius et al., 2010). *Echinacea angustifolia* has a sporophytic self-incompatibility system that prohibits self-fertilization and cross-pollination between closely related individuals that share S-alleles (de Nettancourt, 1997). It is pollinated primarily by native solitary generalist bees (Wagenius and Lyon, 2010).

An *E. angustifolia* plant typically first flowers when it is 3–5 years old. A plant may not flower each subsequent year; instead it persists as a basal rosette. An *E. angustifolia* population generally blooms from late June to mid-August. A flowering plant usually has 1 or 2 heads (capitula), although it can have more than 10. Each flowering head consists of a row of sterile ray florets, each of which produces a ligule (petal) and then 100–300 uniovulate disc florets (hereafter florets) arranged in regular circular rows. The anthers in each row of florets shed pollen on the same day. A style emerges through each floret the day after pollen is shed. The florets develop regularly up the flowering head; bottom row florets shed pollen first, and the top rows of florets shed pollen last.

Study site—This study was conducted during one season (2005) in western Minnesota (near 45°49′ N, 95°43′ W) in an experimental plot previously established as part of a fragmentation study (Wagenius, 2004). The plot is a former agricultural field containing *Solidago rigida, Bromus inermis,* and *Medicago sativa*, among other flowering plants. Native prairie grasses have been reintroduced. Most of the *E. angustifolia* flowering in 2005 were collected as seeds from 1995–1998 from 19 remnant prairie sites within 5 km of the experimental plot. Seedlings were planted on a grid (40 m × 73 m). Density of flowering plants varied in the plot because some plants died, and reproductive age plants do not flower every year (Fig. 1). Of the plants that flowered in 2005, 107 maternal lines from the remnant prairie sites were represented with an average of 1.7 flowering plants per maternal line (range 1–6). There were also 20 flowering plants with unknown maternal lineage. Our experimental plot resembles nearby remnant *E. angustifolia* populations in features such as topography, population size, density of flowering plants, the surrounding landscape, and the community of pollinators. The diversity of co-flowering plants varies considerably among populations, and our experimental plot is less diverse than average. Pollination visitation rates are comparable to the remnants; however, seed set in the common garden is higher than in many remnants; likely because mating incompatibilities are reduced compared to some remnants (Wagenius et al., 2007). In addition, 19 plants flowered in an experimental plot 247 m away. The nearest remnant population with flowering plants in 2005 was approximately 420 m away and contained approximately 100 flowering plants. The next nearest remnant was 520 m away and contained four flowering plants.

Field and germination methods—We followed daily flowering phenology for each flowering head and recorded the following information: (1) first date that at least one floret shed pollen, (2) number of florets shedding pollen, (3) last date that a floret shed pollen, and (4) date that all styles had shriveled and were no longer receptive (Fig. 2). Based on these data, we also calculated the potential for phenological assortative mating using the methods described in Weis and Kossler (2004). We calculated ρ (Weis and Kossler's Eq. 2), which is the phenotypic correlation of flowering time between potential mates. We calculated ρ three different ways using start date, end date, or peak flowering date. Six plants had incomplete flowering phenology data.

We collected and dried leaf samples from nearly every flowering plant in the experimental plot (two plants were not sampled because of disease, and were unlikely to sire offspring), and from all 19 plants in the neighboring plot (N = 221). To cover the span of a plant's flowering, we sampled 10 fertilized achenes (fruits) from each of three groups per maternal plant (30 total): florets that shed pollen within the first three days of a plant's flower phenology, florets that shed pollen within the last three days, and a random sample of the remaining fertilized achenes. We aimed for 30 fertilized achenes per maternal plant although some

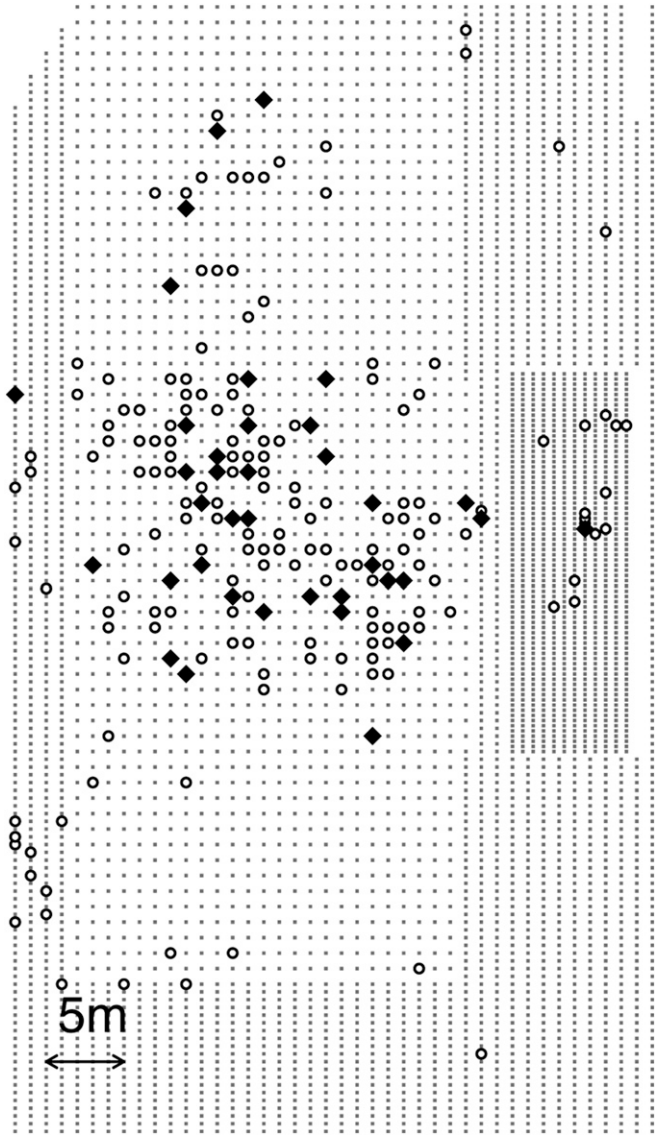

Fig. 1. Map of the experimental plot. Open circles and black diamonds indicate flowering plants ($N = 204$; includes the two diseased plants). Black diamonds indicate the 37 maternal plants from which offspring were used for paternity and pollen pool analysis. Small points represent all planted positions; plants were spaced on either a 1×1 m grid, a 1×0.5 m grid, or a 0.67×0.33 m grid.

had fewer. The fertilized achenes were germinated using the protocol of Feghahati and Reese (1994) as modified by Wagenius (2004) and grown in plug trays until the first true leaf could be sampled (about a week). Achenes that did not germinate were cut open to see if they contained an embryo. Out the achenes that contained an embryo, 93% germinated and survived until sampling. A total of 927 offspring were genotyped in this study; all 37 maternal lines had at least 5 offspring, and 30 of them had 20 or more offspring.

***Paternity assignment*—**To assign paternity to seeds and characterize pollen diversity for each maternal plant, we used 11 polymorphic microsatellite markers developed for *E. angustifolia* (Ison et al., 2013; GenBank accession numbers KF150005-14). For the plants in this study, the microsatellite loci had 3–15 alleles, and a combined paternity exclusion probability of 0.9994. The combined allelic dropout (ε_1) and genotypic mistyping (ε_2) error rate was estimated to be 0.01 across all loci (calculated from maternal-offspring mismatches). For DNA extraction and genotyping protocols, see Ison et al. (2013).

Paternity was assigned using the maximum likelihood method implemented in CERVUS 3.0.3 using Delta scores (Kalinowski et al., 2007). Delta is the difference in the LOD (natural log of the overall likelihood ratio) of the most likely and second most likely candidate sire. Critical Delta scores at different confidence levels are determined by simulation. For the simulation, we used 100 000 offspring as recommended by the CERVUS manual. We also used 240 candidate fathers (of which 221, or 92%, were sampled, allowing for potential gene flow from flowering plants in populations within 500 m). Other simulation parameters included 0.90 as the proportion of loci typed (which is the average proportion typed in our samples), 0.01 as the genotyping error (estimated from maternal-offspring mismatches), and a minimum of six loci typed. Paternity assignments based on Delta scores at both the 95% and 80% confidence level (hereafter 95CL and 80CL, respectively) are presented.

We also used a rarefaction approach to estimate the total number of plants that sired seeds in each of our focal maternal plants. The R package vegan 2.0-5 (Dixon, 2003) was used by adapting the species accumulation function, where our 'species' were sire plants represented in the offspring for each maternal plant, with 10 000 permutations for each maternal plant.

***Pollen pool analysis*—**A pollen pool analysis does not require paternity assignment, but can characterize differences in sires within and between maternal offspring groups through comparing inferred pollen haplotypes (e.g., Smouse et al., 2001; Hardy et al., 2004; Sork et al., 2005). We used two approaches, one compared the similarity in sire sets between maternal plants, and a second estimated the effective number of pollen donors for each maternal plant. Correlated paternity between offspring within a maternal plant (within sib-group) or between offspring of different maternal plants (between sib-groups) were calculated as described by Robledo-Arnuncio et al. (2006) based on the pairwise kinship coefficient (F) of the inferred pollen haplotypes between offspring pairs. If two offspring share a noninbred father, the expected F would be 0.5, while if they do not share a father, F would be 0 (or negative). Correlated paternity is twice the average F values either between offspring within a sib-group or offspring between sib-groups. Correlated paternity was calculated using the KINDIST software (Robledo-Arnuncio et al., 2007). Sib-groups with fewer than 20 offspring were excluded, as were two sib-groups where all offspring had ambiguous pollen alleles at a locus (i.e. maternal plants and offspring were all heterozygous with the same two alleles), which is not allowed in the KINDIST program. This resulted in 28 sib-groups for analysis.

To calculate the effective number of pollen donors, N_{ep}, per maternal plant, we took the inverse of the within sib-group correlated paternity. Results were compared to a direct approach that calculated N_{ep} based on paternity assignments (Smouse and Robledo-Arnuncio, 2005). We calculated direct N_{ep} both with only offspring that were assigned to a pollen donor, and with all offspring including those unassigned to a pollen donor. Each unassigned offspring was assumed to be fathered by a unique plant, which increases the N_{ep} estimates. Comparing the two yields low and high bounds for N_{ep} (Abraham et al., 2011).

***Spatial and phenological analysis*—**For every possible mating pair in the experimental plot, we calculated both the spatial and phenological distance between the maternal plant ($N = 37$) and all other sampled flowering plants ($N = 201$). Two measures of spatial distance were used: (1) the distance between plants in meters, and (2) the rank of the distance between plants. A given sire plant could be the maternal plant's 1st through 201st nearest flowering plant (hereafter k^{th} nearest neighbor).

Two measures of phenological distance were also used. The first is a pairwise synchrony index, which quantifies the synchrony between two individual plants, maternal plant A and sire plant B:

$$\text{sync}_{pw} = \left(\frac{\sum_{d=1}^{j} (A_d B_d)}{\sum_{d=1}^{j} (A_d)} \right)$$

where d is the day of the flowering season, 1 and j are the first and last day, A_d is the number of receptive styles produced by plant A on day d, and B_d indicates that plant B sheds pollen on day d (1 is yes, 0 is no). Thus, if plant B shed pollen every day that plant A has receptive styles, then pairwise synchrony is 1. If plant B sheds pollen for only a few days during which plant A has receptive styles, then the pairwise synchrony is less than 1, weighting each overlapping day by

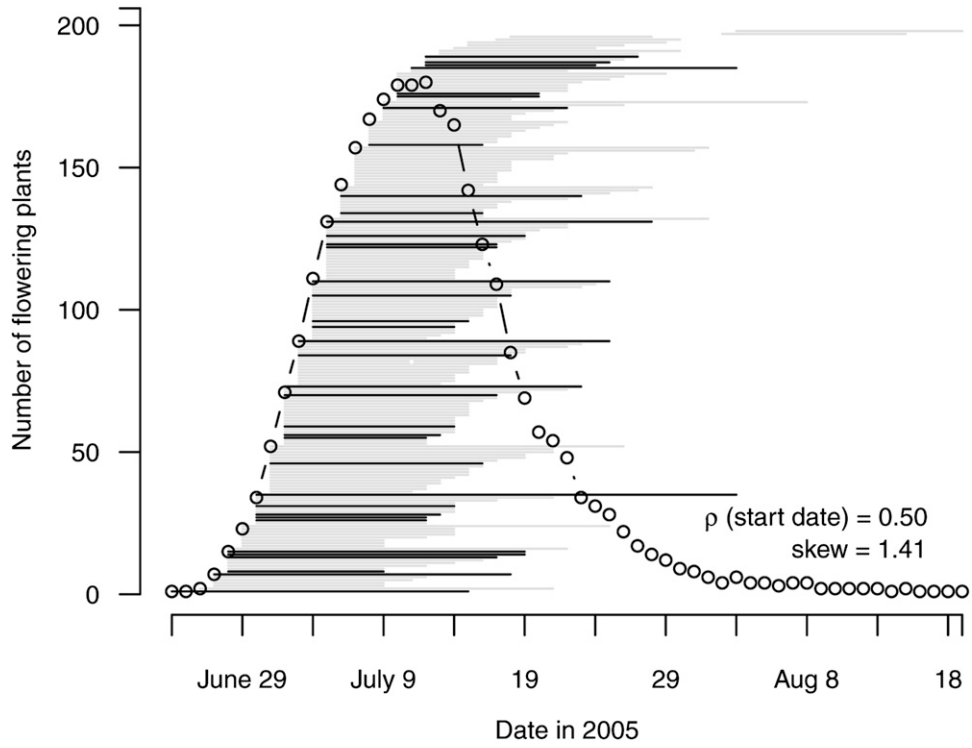

Fig. 2. Flowering schedules for all plants in the experiment plot. Open circles represent the total number of plants flowering each day. The horizontal segments represent the flowering duration of each plant in the experimental plot, and the black segments highlight the 37 maternal plants used in this study.

the number of styles on plant *A*. This is a pairwise synchrony measure because it quantifies the synchrony of two plants to each other. Other synchrony measures estimate synchrony of an entire population, or of an individual plant to the population (reviewed in Bolmgren, 1998; Bjørnstad et al., 1999; Elzinga et al., 2007). The second measure is the absolute value of the difference in peak flowering dates between two individual plants. We calculated peak flowering date for each plant as the mean date of flowering weighted by the number of anthers shedding pollen on each flowering day.

We compared distributions of observed matings to random mating using two spatial predictors (distance between plants in meters and the k^{th} nearest neighbor) and two phenological predictors (pairwise synchrony and difference in peak flowering dates). Random mating is the distributions of all potential mate pairs: focal maternal plants with all potential mates. We tested for deviation from random mating first using the nonparametric Kolmogorov-Smirnov test (K-S test), which tested if the two frequency distributions differed. In addition, to quantify and compare deviation from random mating, we calculated χ^2 goodness-of-fit for the two spatial predictors and phenological predictors, with random mating as the expected values. Different numbers of bins (5– 25) were tested to see if bin size affected the results.

We tested the independence of spatial and phenological measures of isolation on the probability of mating, using generalized linear models (GLMs) with a binomial response of pairwise mating (1 for at least one observed mating between maternal and sire plant, and 0 for no observed mating between maternal and sire plant). We also conducted this analysis with the total observed matings for each maternal and sire plant pair; for this, we used GLMs with a negative binomial distribution (R package MASS version 7.3-22; Venables and Ripley, 2002). For all GLMs, we used one spatial, one phenological, and a spatial x phenological interaction term in each model. Each model was compared with the interaction term to the nested additive model. To assess the consistency of the spatial x phenological interaction, we tried all combinations of spatial (distance in meters, and k^{th} nearest neighbor) and pairwise phenological predictors (pairwise synchrony, and difference in peak flowering dates). This analysis was also conducted using seasonal phenological predictors such as flowering start date of the maternal plant. All combinations of spatial and seasonal predictors (start, peak, and end date) were tried. We tested a quadratic term in the models with a seasonal predictor. There was no evidence for a unimodal relationship between the seasonal predictor and mating probability, as evidenced by a nonsignificant quadratic term ($P > 0.1$; data not shown).

To see if maternal plants were sampling the same pollen pool, we examined the extent to which pollen pools were similar between maternal plants using between sib-groups correlated paternity values. Using linear models, we tested if similarity in pollen pools could be predicted by either distance between plants, pairwise synchrony of the plants, or difference in peak flowering dates between maternal plants (*P*-values were Bonferroni corrected). All analyses not previously specified were conducted using R 2.12.2 with the base package stats (R Development Core Team, 2011).

RESULTS

Echinacea angustifolia flowered from 24 June to 19 August, with the most plants (176) flowering on 11 July (Fig. 2). Individual plants flowered 14 ± 4 d (mean ± standard deviation; $N = 204$ plants, including the two diseased plants). The distribution of flowering plants per date was positively skewed ($P < 0.001$). The phenotypic correlation of flowering time between potential mates (ρ; Weis and Kossler, 2004) indicates that enough variance exists in flowering phenologies between the plants to allow for phenological assortative mating (ρ = 0.50, 0.59, and 0.42 based on start date, peak date, and end date, respectively).

Paternity was assigned to 832 of 927 offspring at 80CL and 487 at 95CL. Of these assignments, 22 (80CL) and nine (95CL) were assigned to plants in the nearby plot. An additional five (80CL) and two (95CL) were assigned to plants with incomplete flowering phenology data. We removed these and used the 805 (80CL) and 476 (95CL) remaining assignments for the within experimental plot analysis. We had 2% and 6.8% of the genotypic data missing across loci, for flowering plants and offspring, respectively.

Mating pairs were closer and more synchronous than expected under random mating (mean observed intermate distance 8.34 m or 9.90 m, expected = 16.0 m; mean observed flowering synchrony = 0.778 or 0.804, expected = 0.670, based on 95CL and 80CL, respectively). A total of 183 of the 201 plants (91%) were assigned as a sire to at least one seed from the 37 maternal plants (80CL). A single pollen donor sired, at most, eight offspring in a sib-group (Appendix S1, see Supplemental Data with the online version of this article). We found a mean of 16.3 assigned sires (range: 3–25) per maternal plant, and a mean of 18.8 sires in sib-groups where paternity was assigned to at least 20 offspring ($N = 29$). Inspection of the rarefaction curves indicates that most of the 37 maternal plants likely had more pollen donors than identified in our sample of offspring (Appendix S2).

Distances between mating pairs were much closer than expected under random mating. but with a tail that extended the entire distance of the experimental plot. Approximately two-thirds of the observed pollinations were between plants 10 m apart or less (62% 80CL and 70% 95CL), and over a third were between plants less than 5 m apart (33% 80CL and 40% 95CL). The distribution of observed pollination distances was left-skewed compared to the distance distribution of all flowering plants in the plot to the maternal plants (Fig. 3A; K-S test: $D = 0.348$ for 80CL, and $D = 0.430$ for 95CL, both with $P < 0.0001$). Neighboring plants mated more often than expected under random mating (Fig. 3B, K-S test: $D = 0.336$ for 80CL, and $D = 0.416$ for 95CL, both with $P < 0.0001$). Matings between plants that were 1st through 20th nearest neighbors accounted for 46.2% (95CL; 38.5% 80CL) of observed pollinations, compared to the 10% that was expected under random mating.

Flowering synchrony between mating pairs was also much greater than expected under random mating. Of the observed crossing events, over 40% had a pairwise synchrony between 0.9 and 1, but only 26% of all potential mating pairs had pairwise synchronies this high (Fig. 3C; K-S test: and $D = 0.185$ for 80CL, and $D = 0.231$ for 95CL, $P < 0.0001$). Plants with more similar peak flowering times were also more likely to mate (Fig. 3D; K-S test: $D = 0.156$ for 80CL, and $D = 0.184$ for 95CL, $P < 0.0001$).

Plants deviated from random mating by spatial distance and phenological similarity according to a χ^2 goodness-of-fit test, regardless of the number of bins (5–25) we used ($P < 0.0001$; Fig. 4). Observed mating deviated from random mating expectations more by location (k^{th} nearest neighbor $\chi^2 = 1001.0$; distance $\chi^2 = 857.0$) than by pairwise synchrony ($\chi^2 = 171.5$) or difference in peak flowering dates ($\chi^2 = 91.7$; all 15 bins, paternity assigned at 80CL).

The probability that a pair of plants mated depended on the distance separating them and their flowering synchrony. However, the magnitude of the effects varied by distance and synchrony, as evidenced by a significant isolation x phenology interaction term in all GLMs tested ($P < 0.05$, Table 1, Appendix S3). Plants with little flowering overlap (synchrony = 0.1) had a 3.5–6% probability of mating (expected number of offspring 0.06–0.01) regardless of their distance apart (Fig. 5A, Appendix S3). In contrast, matings among pairs with average or higher synchrony varied strongly with distance; synchronous pairs less than 5 m apart were four times more likely to mate than synchronous pairs more than 22 m apart (19–28% compared to < 5%, Fig. 5A).

When the probability of mating was examined via a seasonal predictor (maternal plant flowering start date), instead of a pairwise synchrony, we found that pollination distance varied across the season (Fig. 5B, Appendix S3). The magnitude of the effects varied by time in the flowering season and distance, as evidenced by a significant distance x phenology interaction term in all GLMs tested ($P < 0.05$, Table 1, Appendix S3). Pollen movement was the most affected by distance in early flowering plants; plants that were nearby had about a 17% probability of mating (expected number of offspring = 0.48), while the most distant plants had about a < 1% probability of mating (expected number of offspring ≈ 0; Fig. 5B, Appendix S3). For late flowering plants, probability of mating only dropped from 11 to 3% between the closest and farthest plant pairs (Fig. 5B). Late flowering plants had a higher probability of mating with a plant > 20 m apart than did an early flowering plant (Fig. 5B, Appendix S3).

The effective number of pollen donors per maternal plant, as calculated from within sib-group correlated paternity, ranged from 4.3 to 118.3 (mean 23.7; Appendix S1). Directly estimated N_{ep} using paternity assignments yielded similar results; mean N_{ep} was 23.3 when unassigned offspring were excluded and 29.0 when unassigned offspring were included and assumed to have unique sires.

Despite the influence of distance and phenology, each maternal plant mated with a nearly unique set of pollen donors. The mean between sib-groups correlated paternity was ~ 0 with a range of −0.13 to 0.11, meaning that few maternal plants sampled the same group of pollen donors. In fact, most (76%) of the pairwise correlated paternities were below 0.02. We also found that neither nearby nor phenologically similar maternal plants sampled a similar set of pollen donors. Spatial and phenological predictors did not significantly predict the correlated paternity values between maternal plants ($P > 0.05$, Bonferroni corrected).

DISCUSSION

Assortative mating by flowering time—Our study addressed the relative importance of spatial distance and flowering phenology for explaining mating patterns in *E. angustifolia*. Pollinations are often predicted to be predominantly between neighboring plants (Levin and Kerster, 1974). Plants will also mate assortatively based on their flowering time (e.g., Fox, 2003; Weis and Kossler, 2004), but this component of pollination has received much less attention than distance. To date, estimates of phenological assortative mating have been conducted without paternity assignment, but rather by parent-offspring regressions and through examining the phenotypic correlation of flowering time among potential mates (Weis and Kossler, 2004; Weis, 2005). By using paternity assignment to measure the flowering synchrony and distance between parents of hundreds of seeds, ours is one of the first studies to directly and simultaneously measure the role of both parameters in explaining plant mating patterns (Gérard et al., 2006).

Increased mating between synchronous plants does not necessarily demonstrate phenological assortative mating. For instance, an early flowering pollen donor with a long flowering duration may be completely synchronous with a late flowering maternal plant; however, their mating would not represent phenological assortative mating. Therefore, we used the difference in peak dates between observed and potential mates. High potential exists for assortative mating based on peak dates ($\rho = 0.59$). For about a third of the crossing events, peak flowering between the pair differed by two days or less, much less than

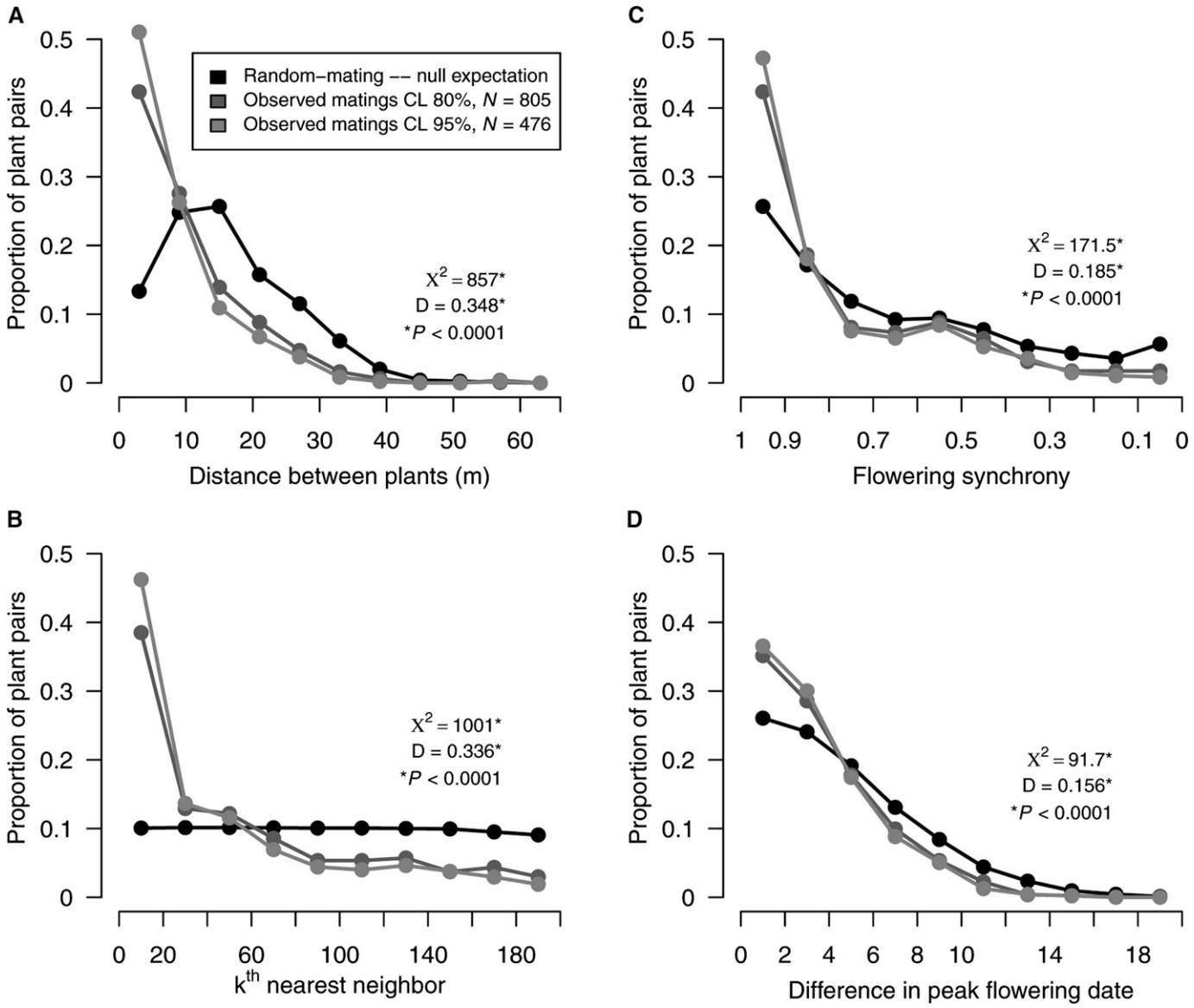

Fig. 3. Deviation of observed mating events from random mating by distance between plants in meters (A), by the k^{th} nearest neighbor of plants (B), by pairwise synchrony between plants (C), and by the difference in peak flowering dates between plants (D). Random mating (black line) is the actual proportion of all flowering plants in the experimental population from the 37 maternal plants. The other lines are the proportion of observed pollinations using paternity assignment at 80CL (darker gray line) or 95CL (lighter gray line). The χ^2 values (15 bins) and *D*-values (K-S test) are on each plot (80CL).

average, demonstrating phenological assortative mating, although not as much as we might expect based on the high ρ value. This is likely because ρ, the phenotypic correlation of flowering between plants, does not take into account distance between plants. We found that distance between plants has a larger effect on which plants mate than the plants' flowering time (Figs. 3 and 4). Thus, while phenological assortative mating is occurring in *E. angustifolia,* its impact is moderated by influence of distance on mating patterns.

Even with only low levels of phenological assortative mating, temporal genetic structure can occur as long as flowering time is heritable (Hendry and Day, 2005). Such temporal genetic structure was recently reported in tropical trees (Dainou et al., 2012). The offspring in our study are part of an experiment in progress that will estimate heritability of flowering phenology; the parental plants are part of an ongoing experiment that quantifies temporal genetic structure within and between prairie remnants.

Flowering synchrony and male fitness—Among neighboring plants, we found that pairwise synchrony did predict mating probability (Fig. 5, Appendix S3). This is an interesting finding in the context of selection on flowering phenology. A recent meta-analysis found strong selection for early flowering time, but weak and inconsistent selection for flowering synchrony (Munguía-Rosas et al., 2011). However, only 5 of the 87 studies in the meta-analysis examined male fitness (all of the studies examined female fitness). We similarly found enhanced seed

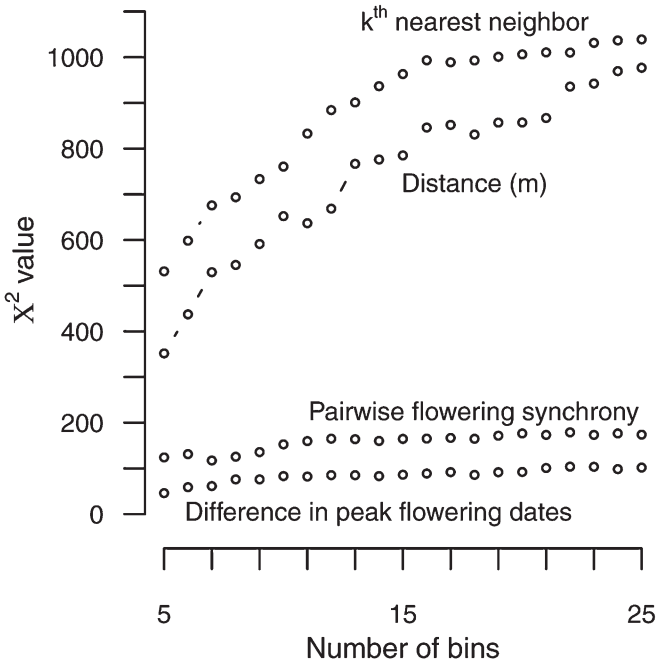

Fig. 4. Comparing deviance from random mating among spatial and phenological predictors. The χ^2 goodness-of-fit values of observed matings to expected values based on random mating, were used to quantify the nonrandomness of observed matings. Regardless of the number of bins used (5–25) in the contingency table, all spatial and phenological predictors deviated from random mating expectations ($P < 0.0001$). Refer to Fig. 3 for observed matings compared to random mating expectation.

set (female fitness) in early flowering plants regardless of flowering synchrony (Ison, 2010). While our current study does not estimate male fitness, the overall mating patterns indicate that synchrony is likely important for male fitness in *E. angustifolia*. Additional studies on flowering synchrony could determine if selection is acting through male fitness rather than female fitness.

***Pollen movement distances*—**We found that intermate distance was more closely associated with mating probability than phenological predictors (Figs. 3 and 4). However, matings were not necessarily among nearest neighbors. Over half of the matings occurred between plants separated by more than 20 closer plants. We emphasize that in our experimental plot, mating compatibility rates are high and not correlated with intermate distance; therefore, we can readily detect pollen movement to a receptive stigma. In contrast, spatial genetic structure and high incompatibility rates in many nearby remnants result in pollen movement without fertilization. Thus, the patterns we observe likely reflect actual pollen movement resulting from pollinator foraging and grooming behavior. For instance, efficient grooming in bumblebees causes pollen from a given flower to only be available to pollinate the next few sequential flowers visited in a foraging bout (Holmquist et al., 2012). *Echinacea angustifolia* is pollinated primarily by a diverse community of native solitary bees (Wagenius and Lyon, 2010), and our results demonstrate that the foraging and/or grooming behavior of these solitary bees leads to a high proportion of pollen movement between plants that are not near neighbors. While this study does not focus on pollen immigration, we also found evidence that solitary bees move pollen between remnant prairie populations. We observed that at least 2% of the pollinations (22 out of 927 offspring) came from a plot of flowering *E. angustifolia* that was 247 m away.

***Pollen movement distances by flowering synchrony and time*—**Our study provides a precise quantitative assessment of how mating probabilities vary based on pairwise spatial isolation and flowering synchrony over time (Table 1, Fig. 5, Appendix S3). The distance between plants was strongly associated with mating probability among plants that were highly synchronous. When flowering overlapped little, mating was rare and varied only slightly with distance (Fig. 5A, Appendix S3). Interestingly, flowering time (early, peak, or late) was strongly associated with pollination distances (Fig. 5B, Appendix S3). Overall, there was a lower probability of mating for late flowering plants, likely due to fewer plants flowering late in the season. Areas with more flowering plants are frequently associated with higher pollinator visitation rates than areas with fewer flowering plants (Fig. 5B; Schmitt, 1983; Kunin, 1997; Grindeland et al., 2005). However, we also observed that late flowering plants had a higher probability of mating with plants from farther away than early or peak flowering plants (Fig. 5B). A similar result is reported for an understory herbaceous plant; pollen from early and late flowering plants moved more than 10 m farther than pollen from peak flowering plants (Kitamoto et al., 2006). One potential explanation is that fewer plants flower late in the season, so pollinators fly farther between plants (Levin and Kerster, 1969; Schmitt, 1983; Fenster, 1991). If changing flowering plant density was indeed the explanation, we might also expect to see an increase in pollination distances early in the season when fewer plants flower compared to peak; however, this was not observed (Fig. 5B, Appendix S3). Therefore, it seems that pollen movement distances vary over the season for reasons other than just changing flowering plant density; perhaps changes in the pollinator community also have an influence.

TABLE 1. Analysis of deviance table comparing GLMs of observed matings, using stepwise model simplification via backward elimination. All models used a binomial response: 1 for a least one mating observed, and 0 for no matings observed. Each additive model had two predictors: one spatial and one phenological. Each full model included an interaction term. The interaction term was tested by comparing the full model with the additive model. In all models, the interaction term was significant. The estimates of the response, i.e., mating probability, from the minimal adequate models are shown in Fig. 5. GLMs with number of observed matings as the response can be found in online Appendix S3.

Model	Model DF	Model Deviance	Test DF	Test Deviance	Test *P*-value
dist + sync + dist x sync	7285	3820.2			
dist + sync	7286	3830.9	1	10.68	0.001
dist + startd + iso x startd	7285	3843.4			
dist + startd	7286	3863.4	1	19.8	< 0.001

Notes: dist = distance in meters; sync = pairwise flowering synchrony; startd = start date of maternal plant.

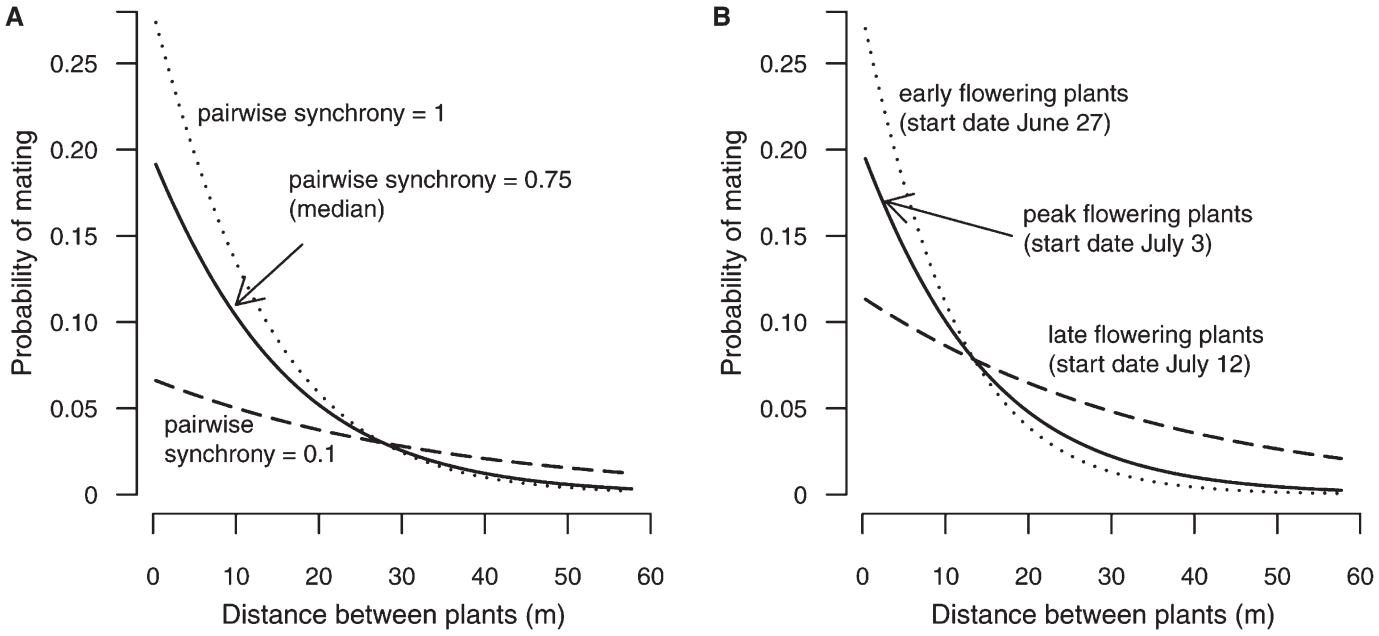

Fig. 5. Interaction of spatial isolation and timing of flowering on mating potential. The *x*-axes show the distance between potential mates in meters. The *y*-axes show the probability of mating between plants based on predicted values from the GLMs (Table 1). In panel A, mating probability is predicted over the range of pairwise flowering synchrony: the short dashed line indicates complete synchrony; the solid line indicates median synchrony (0.75); and the long dashed line indicates low synchrony (0.1). In panel B, mating probability is predicted over the range of flowering start dates: the short dashed line is the date when 5% of all plants had started flowering; the solid line is the date when 50% of plants had started flowering; and the long dashed line is the date when 95% of plants had started flowering.

Generalist pollinated plants can have pollinator communities that change over the course of the flowering season (Herrera, 1988; Cane and Payne, 1993). Foraging behavior may vary among species, resulting in changes in pollen transport (Greenleaf et al., 2007; Mitchell et al., 2009). Lastly, coflowering species also change across the season, and that has been shown to alter generalist pollinator foraging patterns (Kudo, 2007; Mitchell et al., 2009).

***Plants had a large and diverse set of sires*—**Using categorical paternity assignment, we found a mean of 18.8 sires per maternal plant among those with at least 20 assigned offspring (Appendix S1). In addition, the rarefaction curves suggest that the actual number of pollen donors per plant is likely much higher (Appendix S2). This is particularly surprising given the relatively high proportion of offspring that we sampled, nearly 20% of the fertilized achenes produced per plant. While this is much higher than most other paternity studies, we nonetheless missed pollen donors.

Because we could not determine the total number of pollen donors, we instead calculated the effective number of pollen donors (N_{ep}) for each maternal plant. The effective number of pollen donors takes into account evenness in the number of offspring sired by each pollen donor. The effective number of pollen donors varied greatly between maternal plants, but both direct and indirect methods consistently estimated a mean of 23–29 effective pollen donors per maternal plant. Most studies report values of N_{ep} less than 10 (Sork et al., 2002; Irwin et al., 2003; Hardy et al., 2004; Bittencourt and Sebbenn, 2008; Eduardo et al., 2008; Wang et al., 2008), but N_{ep} in some tree species has been estimated to be over 70 (Robledo-Arnuncio et al., 2004b; Abraham et al., 2011). Like these tree species, *E. angustifolia* produces seed crops that are extremely diverse, at least in populations where many compatible pollen donors are available. A pollen pool analysis also demonstrated that maternal plants mated with a diverse and unique set of plants; pairwise correlated paternities were very low. This is in contrast to other studies, which found that nearby maternal plants had highly correlated paternities (Hardy et al., 2004; Robledo-Arnuncio et al., 2004a). At our study site, maternal plants—even those that were very close (less than 5 m apart) and completely synchronous—received pollen from a distinct set of plants. These results indicate that although distance and synchrony influence pollination patterns, the two factors combined explain only a modest portion of the variation in mating patterns.

***Implications for natural populations*—**The pollination patterns that we quantified in the experimental plot give us insight into the pollination patterns in remnant *E. angustifolia* populations. Small *E. angustifolia* remnants often suffer from reduced reproductive success compared to larger remnants; however, reproductive failure is not associated with reduced pollinator service (Wagenius, 2004, 2006; Wagenius and Lyon, 2010). One potential explanation for this is that pollen is moving between incompatible mates. In fact, incompatibility rates are often much higher in smaller remnants compared to larger populations, and near-neighbor plants are more likely to be incompatible (Wagenius et al., 2007). Conducting this study in an experimental plot enabled us to measure pollen movement without the confounding effects of spatial genetic structure. Our results suggest that much of the pollen movement in these small remnants is between incompatible plants, and this, at least in part, explains the reduced reproductive success without reduced pollinator visitation. However, we also found that a large portion of the

pollen movement is from distant plants and that a plant receives pollen from many pollen donors. This suggests that while pollination rates are lower in small remnants, the pollinations that do occur may result from a diverse set of pollen donors.

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