Evidence of hybridization between native *Echinacea angustifolia* and *Echinacea pallida* in a Minnesota prairie fragment

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Overview

Echinacea angustifolia (Asteraceae), the narrow leaf purple coneflower, is the only species of *Echinacea* native to Minnesota tallgrass prairie. Because 99% of the tallgrass prairie has been lost, *E. angustifolia* now survives in small prairie fragments. Recently, a non-native Echinacea species, *E. pallida*, has been introduced to some tallgrass prairies. While *E. pallida* may not be invasive *per se*, it is not known if *E. pallida* poses a threat of genetic swamping. It has been shown that *E. pallida* and *E. angustifolia* can be artificially hybridized (Sanford-Long, 2013), and that they have the same pollinators (Blasini, 2013), but it was not known if they hybridize in nature. We have therefore looked at neighboring clusters of *E. pallida* and *E. angustifolia* at the Hegg Lake State Wildlife Management Area in Kensington, Minnesota and examined indicators of hybridization. We analyzed the relationship between seed set and average distance to the 5th nearest flowering head for 3 regions (bottom, middle, top) of our 22 focal *Echinacea* heads according to three hypotheses. We assumed: *Echinacea* heads only mate with other plants of the same species (the *intraspecific* model); *E. pallida* and *E. angustifolia* mate as if they are a single species (the *nonspecific* model); *E. pallida* only mate with *E. angustifolia,* and vice versa (the *interspecific* model). While the intraspecific and nonspecific models yielded nonsensical results, the interspecific model revealed a statistically significant inverse correlation between seed set and distance to the nth nearest flowering head of the opposite species. These results suggest that *E. pallida* and *E. angustifolia* are hybridizing.

I. Background

The introduction of non-native plants species may displace populations of native plants by out-competing them for resources or by reproducing with them, irreversibly altering the genetic pool. In certain prairie restorations, non-native species of *Echinacea* (*E. pallida* and *E. purpurea*)—whose seeds are more common and less expensive—have been introduced to *E. angustifolia'*s native habitat (Blasini, unpublished).

Prior research suggests that the presence of these non-native *Echinacea* plants in prairie remnants could be a cause for concern. Maternal *E. angustifolia* and paternal *E. pallida* have been observed to have the highest reproductive compatibility amongst intraspecific and interspecific crosses for each species (Sanford-Long, unpublished). *E. angustifolia* is also more likely to be compatible with *E. pallida* than the reverse (Sanford-Long, unpublished). In an experiment by Sanford-Long in which intraspecific and interspecific crosses were performed using *E. pallida* and a native population of *E. angustifolia* plants in a restoration plot at Hegg Lake Wildlife Management Area, crosses between maternal *E. angustifolia* and paternal *E. pallida* plants had over a 90% compatibility rate, slightly higher than that of the intraspecific *E. angustifolia* crosses and significantly higher than the nearly 80% compatibility rate of crosses between *E. pallida* plants (unpublished). The lowest compatibility rate was approximately 70%, between crosses of maternal *E. pallida* and paternal *E. angustifolia* (Sanford-Long, unpublished).

In addition to interspecific compatibility, flowering synchrony and the prevalence of many shared generalist pollinators makes hybridization likely and introduces the possibility of genetic swamping of *E. angustifolia* populations (Blasini, unpublished). It has also been shown that certain communities of arthropods can survive only on *E. angustifolia*--though their ability to survive on *Echinacea* hybrids is unknown (English, unpublished). Thus hybridization may have substantial consequences for the native *Echinacea* species as well as the arthropod populations they support (Blasini, unpublished).

Despite this evidence for the potential threats of hybridization, no prior research has investigated whether *Echinacea* in prairie restorations have been hybridizing. Prior research on the influence of proximity and flowering synchrony on reproduction of *E. angustifolia* transplanted into a common garden will be used as a model to predict how these factors may affect the rate of hybridization between the native and non-native species within remnant prairie populations. The following paragraphs highlight relevant research.

1. Space. Plants that are closer have a significantly higher probability of mating than those that are separated by a greater distance (Ison et al, unpublished). In a study by Ison et al., 2014, approximately two-thirds of the pollinations in common garden *E. angustifolia* occurred between plants within a ten-meter radius and more than a third occurred between plants within a five-meter radius.

2. Time. The likelihood of mating between two plants was also found to correlate with synchronous flowering with similarity in peak flowering times proving a significant predictor (Ison et al, 2014). Mean observed flowering synchrony among mating pairs was around 0.8 compared to 0.670 expected under random mating (Ison et al, 2014). We defined flowering synchrony as the existence of overlap in the flowering days of the plants.

3. Space x time. Mating among pairs with average or higher synchrony is strongly influenced by distance (Ison et al, unpublished). Non-synchronous plants had a very low probability of mating despite the distance separating them, while synchronous pairs less than five meters apart were four times more likely to mate than synchronous pairs less than 22 meters apart (Ison et al, 2014). In our spatial analysis, we used isolation as a measure of distance from a plant to its fifth nearest flowering neighbor.

4. Compatibility x space. *Echinacea* species are self-incompatible due to a mate recognition system that prevents self-fertilization and cross-fertilization between close relatives. Lack of reproductive vitality in remnant *Echinacea* populations may also be attributed to limited accessibility of compatible mates. Pairwise compatibility was observed to correlate inversely with pairwise distance in the investigation of 19 remnant populations in which random plants were crossed with their sixth nearest neighbors. More than 90% of pairs separated by over 8.92 m proving compatible compared to only 58% of pairs within 1.77 m (Wagenius, unpublished).

II. The focal species and study site

Echinacea angustifolia (Asteraceae) is an ideal model for this study because it shares many features with other common native prairie plants—it is long-lived, self-incompatible, and pollinated by a wide range of native generalist bees. Thus, the implications of this study can be extended to broader prairie conservation efforts. Large populations of *E. angustifolia* were common before European settlement, but have now been drastically reduced, with some current populations containing only three or fewer individuals separated by over 1000 m (Wagenius, unpublished).

The study area comprises approximately 30,000 square meters of Minnesota's Hegg Lake Wildlife Management Area (HLWMA) near Kensington, MN. The data and *Echinacea* heads were collected by the Echinacea Project from June to August of 2013. The information collected was on populations of *E. angustifolia* and *E. pallida* living in close proximity (Blasini, unpublished). *E. pallida* was introduced 5 years ago into the restoration, which contains a remnant population of *E. angustifolia* (Wagenius, personal communication). No information is known on the genetic background of the *E. pallida.*

III. Work undertaken prior to this study.

In the summer of 2013, Team Echinacea recorded the first and last day of flowering for most of the *Echinacea* heads in the area we examined. Five of the twenty-two heads that are used in the study are missing phenological data. The location of each plant included in our study was mapped by Team Echinacea, accurate to 6 cm. The heads of the plants included in our study were collected late in the summer before their achenes dropped. Each head was bagged individually and catalogued by an identification number unique to each plant, and an identification color unique to each head on a plant.

IV. The work to be undertaken

Hypotheses: Since seed set is directly correlated to the rate of reproduction, we investigated the seed set of *E. angustifolia* and *E. pallida* heads living in close proximity to determine whether they are hybridizing. We examined the seed set from three regions of each head: the bottom, middle, and top. By separating each head into regions, we examined more precisely how phenology affects seed set (each region flowers for 2-5 days, whereas entire heads flower for up 12 days). We tested if the presence of the nearby *E. pallida* population affected the seed set of *E. angustifolia* and vice versa, taking into account the effects of phenological and spatial isolation.

Null hypothesis: Graphs of seed set vs. distance to *n*th nearest flowering head of the same species will be the only graphs with consistent, logical, and statistically significant correlations between seed set and isolation. That is, the intraspecific model will fit the dataset.

Alternate hypothesis (nonspecific and interspecific hypotheses): Graphs of seed set vs. distance to *n*th nearest flowering head, or graphs of seed set vs. distance to *n*th nearest flowering head of the opposite species will be the only graphs with consistent, logical, and statistically significant relationships between seed set and isolation. We speculate that *E. angustifolia* will hybridize readily with *E. pallida*. Therefore certain individual *Echinacea* heads that are isolated from *Echinacea* of the same species, but are in close proximity to flowering *Echinacea* of the other species will display relatively high seed set. Since *E. pallida* and *E. angustifolia* have been shown to pollinate interspecifically more readily than they pollinate intraspecifically, we expect to see the highest seed sets in regions of heads that are in close proximity to flowering *Echinacea* of the opposite species.

Objectives: 1: Predict individual seed set based on both interspecific and intraspecific spatial isolation and flowering phenology. 2: Determine actual seed set of regions from individual *Echinacea* heads. 3: Develop an individual-based model of *Echinacea* seed set based on spatial isolation and temporal isolation from *Echinacea* of the same species, all species, and opposite species.

Methods and Procedures:

First we removed the achenes from the collected heads, separating 30 achenes from the bottom of the head, the 30 from the top, and the remaining middle achenes. We also kept ray achenes, which are sterile, separate. After removing all other floret parts, we placed achenes in envelopes displaying the identification number of their plant, the tag color of their head, and the region they came from (top, middle, bottom, ray). We also had one envelope for achenes of unknown region. We scanned images of the middle achenes and counted the number of achenes. For all sets of middle achenes that had more than 30 achenes, we selected a random subset of 30 achenes and placed those in separate envelopes. We then used an automated weighing apparatus to weigh all the bottom, top, and 30 randomly selected middle achenes. We put all achenes (including the unweighed achenes excluded from the subset of 30 randomly selected middle achenes) in glassine envelopes, which we tagged with a unique identifier of the plant ID, tag color, and region. We x-rayed all these achenes at 12 kV for 4 seconds, and generated digital x-ray images. We then counted how many achenes had embryos within them. Using this count of embryos from the x-rays, the total number of achenes counted on the scans of the middles, and number of achenes removed from the tops and bottoms (30), we determine each head's seed set: the proportion of achenes containing seeds. All achenes remain in their x-ray envelopes so that in future studies will know exactly from which head and region the achenes were removed. We will analyze weight data along side x-ray data in order to determine a weight cutoff for deciding which achenes are full for pallida achenes.

Using our dataset of pairwise distances and flowering phenology, we determined each individual's distance to an k^{th} nearest flowering neighbor. We assumed the heads lacking phenological data flowered for the entire duration of their species' flowering season. We plotted the seed set of each region of each head versus the distance to an n^{th} nearest flowering neighbor three times: once treating *E. pallida* and *E. angustifolia* as incompatible, once assuming they behave as a single species, and once assuming they only mate interspecifically.

Results

Three lines of evidence suggest hybridization occurred at Hegg Lake.

We first looked at a particular region of an *E. angustifolia* head that did not flower synchronously with any other *E. angustifolia* heads (ANG-110-wht-btm). This region did flower in synchrony with many *E. pallida* heads within close proximity. The bottom of that head had the highest seed set of all the partitions of the *E. angustifolia* heads (seed set=0.36), suggesting that hybridization occurred in this head and at a relatively high rate. An alternate explanation would be that the *E. angustifolia* plants are mating with other populations of *E. angustifolia* nearby but not within the study site, however the larger distance the pollinators would have to travel among the populations makes this less likely.

Another point that suggested hybridization is occurring among the plants at Hegg Lake is the possibility of incompatibility among the *E. angustifolia* plants, which would agree with our knowledge of the long length of time the angustifolia population probably spent reproducing with each other. The top (late) portions of the angustifolia heads--whose flowering times were generally very synchronous with other nearby *E. angustifolia* but were generally asynchronous with *E. pallida* plants--had very low seed set (in the 0 to 0.23 range). An *E. angustifolia* plant (ANG-110-wht) whose late flowering time was synchronous with all three other *E. angustifolia*--two of which were only a couple meters away--had almost zero seed set (seed set=0.06). This incompatibility might suggest that the relatively high seed set in the bottom (early) portions of the E. angustifolia heads in comparison to the top (late) portions of those heads was a result of hybridization.

Finally, comparison at the four models also revealed evidence that hybridization is occurring. Mean distance to the 4th nearest flowering neighbor has a good range of data for all models, so we focused on that metric. For the intraspecific model, which assumes mating occurs only between plants of the same species, the graph of *E. angustifolia* seed set vs. mean distance to the 4th nearest flowering *E. angustifolia* neighbor yielded peculiar results (fig. 1).While the top and middle portions of the head had negative slope, which is expected, the bottom portion of the head had very positive slope. Similarly, the graph of *E. pallida* seed set vs. mean distance to the 4th nearest flowering *E. pallida* neighbor yielded an odd graph (fig. 2). The bottom region has positive slope, the middle has negative, and the top has slope near 0. The failure of the intraspecific model to yield negative slopes for all regions suggest that this model is flawed and cannot accurately predict seed set. The data suggests that the *E. angustifolia* and *E. pallida* plants cannot be evaluated alone and nearby synchronous plants of the other species may be accounting for these unexpected trends.

The model that treated all *Echinacea* as a single species, the nonspecific model, also yielded an odd graph (fig.3). For the graph of *Echinacea* seed set vs. mean distance to the 4th nearest flowering *Echinacea* head, the middle and top regions have negative slopes while the the bottom has a positive slope. Thus the nonspecific model fails as well

The only model that resulted in consistent and coherent results was the interspecific model. The graph of seed set vs. mean distance to the 4th nearest flowering *Echinacea* of the opposite species (fig. 4) shows that seed set decreases when heads are isolated from heads of the opposite species.

The failure of the intraspecific model and the success of the interspecific model suggest hybridization is occurring, since the model that assumes hybridization succeeded while the model that denies hybridization fails. However, the failure of the nonspecific model is difficult to explain, since it does take hybridization into account. We speculate that its failure is due partly to the small sample size, and also do to the fact that we did not take compatibility into account. The remnant *E. angustifolia* plants may very well have been incompatible with many of the other *E. angustifolia* plants, since plants living in close proximity to each other are much more likely to share a mother. So there could be another dimension to their isolation we neglected--isolation due to incompatibility with half-siblings. This compatibility dimension would be negligible in the interspecific model, because heads of two different species certainly do not share a mother, even if they live in close proximity

Conclusion

We have conducted a short-term study that generated immediate, preliminary data on the possibility of hybridization between non-native *E. pallida* and native *E. angustifolia*. Our results suggest that the two species do hybridize. Certain plants isolated from plants of their own species, but close to flowering plants of the other species had very high seed set. Additionally, the best model we found for predicting seed set assumed hybridization. Therefore, additional studies that germinate our seeds and test their paternity should be undertaken. The data we have compiled will be invaluable for such a study. Additional studies should also be conducted to test if specialist aphids can survive on hybrid *Echinacea.* If it is found that they cannot survive on hybrids, our study would suggest that the hybridization of *Echinacea* in prairie remnants threatens the survival of these aphids.

The spatial and phenological data for our heads, as well as their seed sets--sorted by region-- has been organized in tables and graphs. Our carefully organized data will be invaluable to the next team, who will conduct paternity testing on them. This team will evaluate our predictions and use our datasets to determine how spatial and temporal isolation influence mating between the two species. Further analysis may also be done relating the vigor of the plants to the weights of the achenes from which they were germinated.

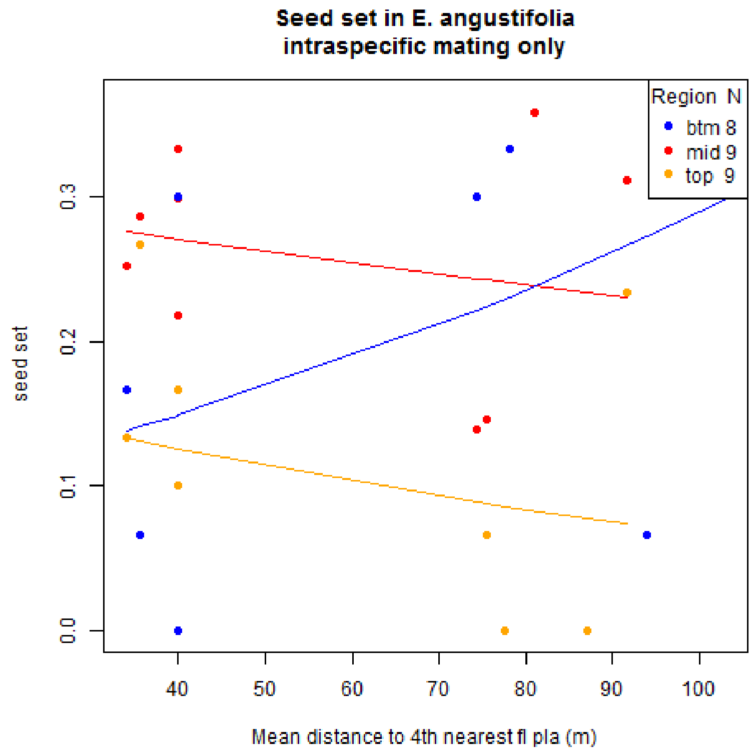

Figure 1

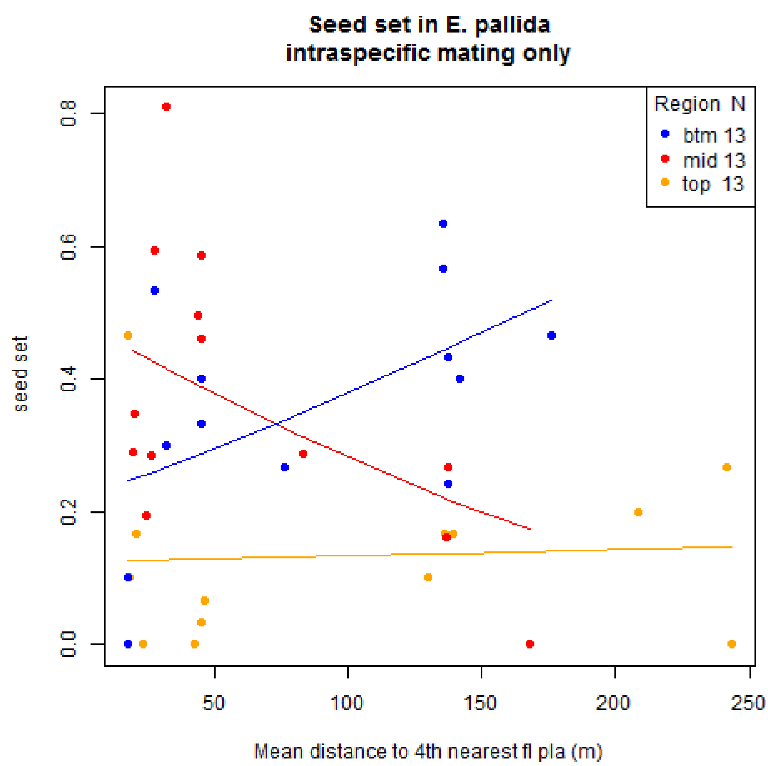

Figure 2

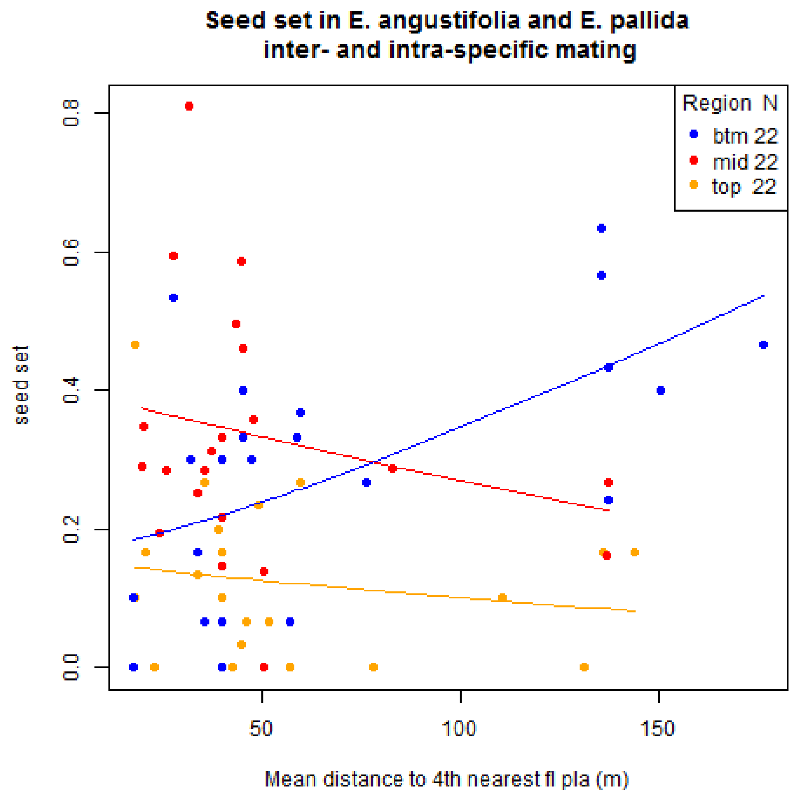

Figure 3

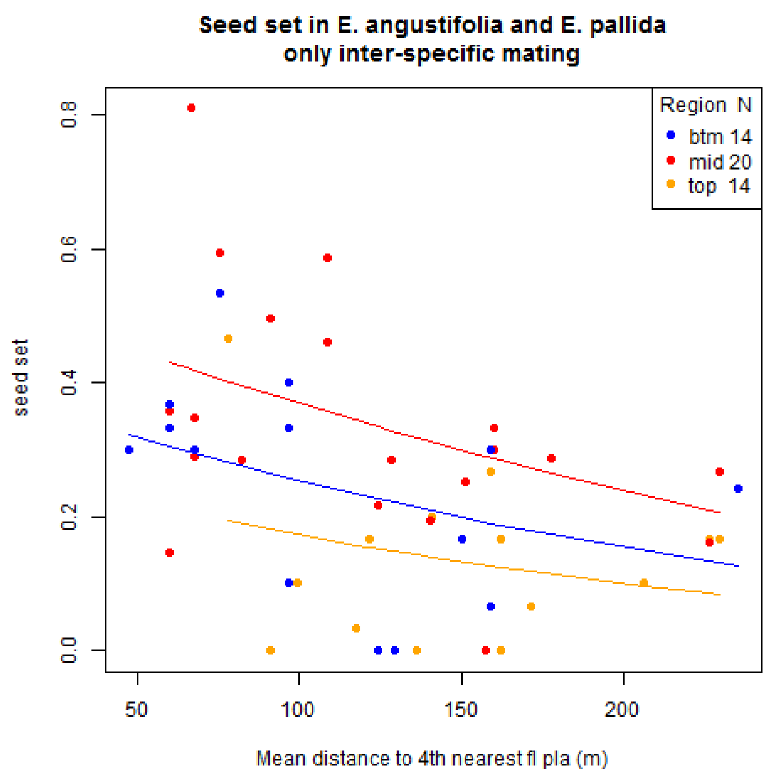

Figure 4