Title: Outcrossing and fecundity in the woodland sedge, *Carex pensylvanica:* implications for ecological restoration

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**Abstract:**

Native plant growers face a host of economic and technical challenges that can limit their ability to produce adequate quantities and diversity of plants, and many ecologically important species are often underrepresented in restoration plantings. An example of this is the woodland sedge *Carex pensylvanica* Lam [Cyperaceae] *--* an herbaceous-layer dominant in dry eastern forests -- which is marked by poor seed yield and germination rates, and is, as such, difficult to produce from seed. It is possible that long-term, self-pollination in many wild populations has resulted in inbreeding depression and reduced seed production and fitness. I tested this hypothesis in a greenhouse experiment where I controlled the breeding system of *C. pensylvanica* through hand-pollination to compare the reproductive output between outcrossed and self-pollinated manipulations. Results showed no effect of the breeding system manipulation on seed weight, but seed set in outcrossed plants was significantly higher (1.65x) than seed set in self-pollinated subjects. Based on these data, I developed models that predicted outcrossing seed set at 4.7 seeds/flower while selfing seed set predicted at 2.8 seeds/flower, supporting the hypothesis that long-term selfing is a significant contributor to the low seed production in this species.  This study demonstrates that manipulating the breeding system of *C. pensylvanica* to achieve increased outcrossing is an effective way to increase seed production which would allow growers to increase the availability of plants for restoration projects.

Tags: Cyperaceae, Pollination, Breeding System, Mating System Native Plants, Seed Set, Pennsylvania Sedge

Nomenclature: USDA NRCS (2017)

**Introduction:**

Over the past several decades, ecological restoration has become an important tool for countering the detrimental effects of anthropogenic environmental degradation and global change (Young 2000). As the discipline has matured, a specialty niche for plant growers providing native propagules for restoration projects has emerged within the plant nursery industry (Booth and Jones 2001). However, plants appropriate for restoration tend to have more complex life histories and growth requirements than the developed cultivars favored in traditional horticulture, and production is often restricted due to a lack of production knowledge among growers (Peppin and others 2010).  Because of this, the plant diversity of restoration projects is often constrained the limited species offerings from native plant growers (Hooper et al 2008).

To restore appropriate levels of biodiversity to degraded sites, native plant producers must expand the diversity of their plant offerings to include ecologically important species that have often been overlooked. Sedges of the genus *Carex* (Cyperaceae) are excellent candidates with which to begin such studies. Comprised of over 2,000 species with nearly global distribution, *Carex* is one of the most morphologically and ecologically diverse plant genera (Ball and Reznicek 2003). *Carex* species are herbaceous layer dominants across a diversity of North American ecosystems, but are often underrepresented in ecological restoration plantings (Handel 2015).

The sedge *Carex pensylvanica* Lam. (Cyperaceae)*,* known commonly as Pennsylvania or Penn Sedge, Early Sedge, Oak Sedge, and Early Oak Sedge, is a widespread species and ground layer dominant in a number of ecosystems in the Eastern United States including dry mesic forest, oak savannas and pine and oak barrens (Voss and Reznicek 2012). This species grows vigorously after disturbance, and has been noted for importance in ecological restoration for its ability to compete with exotic species and buffer against invasion (Mottl and others 2007). There is also horticultural interest in *C. pensylvanica* as a lawn alternative for its grass-like form and shade tolerance (Meyer 2004). While *C. pensylvanica* reproduces readily vegetatively, it is notoriously difficult to obtain adequate quantities of seed due to low seed set (Friedman and Barrett 2009) and low germination rates (Farrer and Goldberg 2011).

The potential for this sedge to be used in horticulture and restoration has prompted research to improve seed production. A study by McGinnis and Meyer (2011) attributed the difficulty in germinating this species to complex dormancy requirements. Through after-ripening, cold stratification and temperature and light treatments, the authors achieved substantially improved germinations rates, but their results varied significantly between the two years of their trials (57-96% germination in year one and 31-67% in year two) in which two different seed stocks were used. The authors suggest that the observed variation between trials may be a result of underlying population or genetic differences between the seed stocks, indicating that to understand the regeneration characteristics of *Carex pensylvanica* as pertaining to native plant production, other aspects of its reproductive life cycle must also be examined.

In plants, seeds can be produced through autogamy (self-fertilization), allogamy (outcrossed fertilization), apomixis (seed production without fertilization) or a combination (Shivanna and Tandon 2014). In all organisms, any factors that determine the patterns of gene inheritance between generations are collectively known as the breeding or mating system (Kearns and Inouye 1993) and for plants, the breeding system is essentially a measure of the relative dependency on outcrossing or selfing for reproductive success. *C. pensylvanica* is a long-lived perennial plant with vigorous vegetative reproduction that produces large mats of genetically identical ramets that can be acres in size (Bernard 1990). Flowers of *C. pensylvanica* are monoecious, with the staminate spike superposed above one or several pistillate spikes (Hipp 2008). Friedman and Barrett (2009) found *C. pensylvanica* to be self-compatible, and at least in their study system, to demonstrate high rates of self-pollination. This finding is consistent with an expectation for a tight relationship between clonal spread and incidences of self-pollination that has been demonstrated in other species (Handel 1985).

Long-term self-pollination can lead to reduced fitness from inbreeding depression (Charlesworth and Charlesworth 1987) and several studies have found the negative effects of inbreeding to be expressed in seed characteristics such as seed set and germination (Kalisz 1989, Keller and Waller 2002, Carta and others 2015, Joschinski and others 2015, Rymer and others 2015). However, other studies posit that highly selfing plants may, over time, purge the deleterious alleles that lead to inbreeding depression (Barrett and Harder 1996). In such cases, outcrossing with genetically distinct individuals may disrupt these stable genetics, resulting in a reproductive diminishment (Schierup and Christiansen 1996).

Low seed set and germination rates, and poor seed fitness characterize sexual reproduction in *Carex pensylvanica*. Evidence in the literature and observations of the physiology of *C. pensylvanica* suggest that it is highly selfing. From this, I hypothesize that 1) *Carex pensylvanica’*s typical poor seed quality is a result of long-term inbreeding through self-pollination and that 2) outcrossing should produce seeds with improved fitness characteristics. To test this hypothesis, I performed a hand pollination experiment, in which I artificially manipulated the breeding system of *C. pensylvanica* clones to produce crops of outcrossed and self-pollinated seeds for comparison. This study has important implications for better understanding the reproductive ecology and evolutionary trajectory of the species as a whole. It also has practical applications for aiding native plant growers in their ability to produce *C. pensylvanica* stocks for ecological restoration.

**Materials and Methods:**

*Sampling and Experimental Setup:*

I obtained all plant material for the study from 10 wild *C. pensylvanica* clones at the University of Michigan’s Matthaei Botanical Gardens and Radrick Forest properties in Ann Arbor, Michigan (42.2994° N, 83.6622° W) in July of 2015. All sampling took place on glacially deposited till, in unmanaged, closed canopy Oak-Hickory woodlands. Sampling was restricted to populations deemed native to the site before it was managed as a botanical garden, and under no influence from the cultivated collections.

I identified clones on a ground survey though the study area. All clones that I used were a minimum distance of 100 m (328 ft) apart from each other and separated by natural breaks such as streams, wetlands, or steep cliffs to maximize the likelihood that the samples came from genetically distinct clones Clones ranged in size from 5-50 m (16-150 ft) in diameter. At each sampling location, I removed 50 plant masses, each 15 cm (6 in) in diameter, from each clone (n=500 total) to a depth of 20 cm (8 in) in order to leave roots intact, and transported them back to the greenhouses of the Matthaei Botanical Gardens where they were potted into 15 cm (6 in) square pots. The potted sedges were grown under ambient outdoor conditions from July-September, and fertilized weekly, until I transferred them to outdoor, subterranean cold frames for pre-vernalization hardening off. In mid-September, I placed all pots in a refrigerator room maintained at 5°C (41°F) for 16 weeks of vernalization treatment.

In January of 2016, I removed all plants from the cold room and transferred them to the greenhouses of the Matthaei Botanical Gardens where they were kept under grow lights set for 8 hour-day length, and with average daytime temperature ranging 18-24°C (64-75°F) to simulate early spring conditions and induce flowering. With 30-40% mortality occurring during vernalization, I randomly assigned the remaining samples to pollination treatments, with 15 sedges per clone assigned outcrossing treatment, 15 sedges per clone assigned selfing treatment, and any remaining plant designated as pollen sources.

*Pollination Manipulation*

I applied the pollination treatments at the initiation of flower development, which began ~4 weeks after release from vernalization cold treatment. For all outcrossing treatments, I removed the developing staminate spike from the floral culm with small surgical scissors. I then bagged the culm with a 10x15 cm (4x6 in) pollen-proof bag (Carolina Biological Supply, Burlington, North Carolina), to prevent contamination through geitonogamy, another form of self-pollination where pollen is transferred between two genetically identical ramets within a single genet. For the self-pollination treatments, floral culms remained intact, and I bagged the full culm to prevent contamination from outside pollen. I separated the treatments into adjacent greenhouses to prevent cross-contamination.

I performed visual assessment of pistil receptivity and pollen daily. When outcrossing treatment pistils were receptive, I collected pollen from flowers of multiple other genets and combined on the rim of 16x100 mm (0.6x3.9 in) glass test tubes (Carolina Biological Supply, Burlington, North Carolina) to randomize pollen donation and control for the effect of male fitness differences on the seed development. I then transferred the pollen directly from the rim of the tube to the receptive pistils. For the selfing treatment, I gathered pollen using the same technique, but from the staminate spike of the focal flower and applied it to the receptive pistillate flowers. Because Pennsylvania Sedge is protogynous, pollen availability and pistil receptivity was often offset temporally on the same culm, and in such cases, I gathered pollen from other attached culms and transferred to the receptive pistils, maintaining the selfing treatment through geitonogamy.

Observations and pollination manipulations continued for 6 weeks from the first flowering episode and concluded in March of 2016. I allowed seeds to mature on the culm for 6 weeks after the pollen transfer and then collected for analysis. I evaluated the reproductive outputs of the trials using two proxies for reproductive fitness: seed set and seed weight.

Seed set is defined as the number of viable seeds (with a developed embryo) per flower. I evaluated seed development visually under a Coddington 10x magnifying lens, and I deemed seeds developed if a rounded embryo was visible inside the seed coat. I obtained seed weight values as an average weight per flower, by weighing each flower’s crop of viable seeds on an XS104 microbalance (Mettler-Toledo, Columbus, OH), and dividing this total weight by the number of seeds per flower.

*Statistical Analysis*

All statistical analysis was performed using the R statistical package. I performed initial comparisons for the treatment effect on seed set and seed weight using Welch Two Sample t-test. Because I sampled multiple flowers from the same clones, I further analyzed seed set with generalized linear mixed models (GLMM) (R Core Team 2014, Bates and others 2014) to account this nested design, and account for the influence of clone of origin on the response variable.  I fit the final model to accommodate a poisson distribution typical of count data. Because the seed weight data were normally distributed, I further analyzed seed weight with a linear mixed model (Bates and other 2015) to account for clonal influence on the response variable. I considered the pollination treatment as the fixed-effect variable and clonal origin as the random-effect variable and utilized restricted maximum likelihood (REML) to characterize the covariance between responses of individual plants. I then used the models to determine predicted values for both average seed weight and seed set (R Core Team 2014).

It should be noted that I only included flowers that set one or more seeds in the statistical analysis because it was not possible to evaluate whether the failure of pollinated flowers to produce seed entirely was a result of a biological mechanism, which would have been fit for inclusion in analysis, or a result of experimental error, for example mistimed pollen transfers, which would have been unfit for inclusion. I attempted pollen transfers on 210 floral culms, and based on the aforementioned seed development criteria, I included 121 culms and 174 individual flowers (90 outcrossed and 84 selfed) in the analysis.

**Results:**

*Seed Set:*

Seed set in outcrossed flowers was significantly higher than in self-pollinated flowers (outcrossed mean= 4.7 SE= 0.30, selfed mean= 3.4 SE=0.36, P=0.0068). Based on the generalized linear mixed models, the predicted value for seed set for the outcrossing treatment was 4.6 seeds per flower and the value predicted for the selfing treatment was 2.8 seeds per flower. The range of seed set in this experiment was between 1 and 19 seeds per flower.

*Seed Weight:*

There was no significant difference between the average seed weight of outcrossed and self-pollinated progeny (mean outcrossed=1.12 mg, SE= 0.031, mean selfed= 1.14 mg SE=0.037, P=0.748). The predicted values generated by the linear mixed model were similar to the means of the raw data: 1.14 for selfed treatments and 1.12 for outcrossed. The range for average seed weight was between 0.2 and 2.33 mg.

**Discussion:**

In this experiment, I found no significant effect of breeding system on seed weight. My findings are consistent with observations in the literature that mean seed size is relatively invariant across a given species, especially when seeds are produced under similar environmental conditions (Silvertown 1989).

The higher seed set resulting from outcrossed flowers of *C. pensylvanica* when compared to the self-pollination treatmentsuggests inbreeding depression from long-term selfing as a likely cause of low seed set. While few other studies examine the breeding system of this species directly, the results of this experiment are consistent with Friedman and Barrett’s (2009) study, which found that *C. pensylvanica* culms that had been bagged for selfing set significantly less seed than open-pollinated stems.

These results indicate that manipulating the breeding systems of *C. pensylvanica* to favor outcrossing may be a useful approach to increasing seed production, despite observed seed set being relatively low in both treatments. As is consistent with expectations for plants that have been vernalized to accelerate the annual reproductive cycle, the sedgesstudied in this experiment displayed several irregular floral morphologies including pistillate spikes intermingled or superposed above staminate spikes, or staminate spikes lacking entirely (Vonk Noordegraaf and Welles 1995, Anton Reznicek personal communication). While I confirmed that both male and female flowers were viable and receptive even in these arrangements, the full effect of these anomalies on seed development is unknown. Because these altered morphologies were equally pervasive across both pollination treatments, the comparative results between treatments are sound, but caution should be used when considering the absolute values for seed set obtained in this experiment in comparison to wild populations with more characteristic floral morphologies. While multi-year studies are general preferred in ecology, the controlled experimental conditions of this greenhouse study were designed to homogenize the growing conditions, environmental factors that typically result in inter-annual variability in ecological studies. This controlled setting allows for confidence in this study, however, it should be noted that the theory surround inbreeding depression addresses effects of long term selfing over multiple generation. My study implicates inbreeding depression as the mechanisms reducing seed set in *C. pensylvanica,* but further study over multiple generations would be needed to confirm this implication.

The results of this study indicated the breeding system was an important determinant of seed set and should be considered in *C. pensylvanica* nursery production. The model projected a 1.65x increase in seed set when outcrossing was compared to selfing. On a production scale, this difference could significantly affect a grower's ability to economically produce *C. pensylvanica* propagules. While there is a general preference towards wild collected restoration propagules in order to prevent the inadvertent but inevitable breeder’s selection on cultivated materials (Schröder and Prasse 2013), the high selfing rates for *C. pensylvanica* makes wild collection of seed nearly impossible. Instead, growers might consider constructing seed gardens assembled spatially to maximize the interactions between plants of different genotypes as an option for increasing *C. pensylvanica* seed yield. Such gardens would not only increase the likelihood of outcrossing, but if regularly supplemented, could serve the dual purpose of maintaining a large genetic pool to preserve the adaptive capacity of the plant stock as local conditions change. The economic benefits of this approach and increased seed set for growers would need to be evaluated on a case by case basis, requiring economic analysis beyond the scope of this paper. The utility of this approach may also vary regionally. In this study, I treated the planted materials as representative of the wider germplasm base of the species. To my knowledge, there have been no detailed population genetic studies in *C. pensylvanica* that could serve to evaluate this assumption, but because of the species large geographic and habitat range, it is possible there could be significant differentiation between populations. Future population genetics studies of *C. pensylvanica*, would provide additional insight for growers to consider when evaluating the utility of manipulating outcrossing incidence through seed gardens in the own, local production facilities.

**Conclusion:**

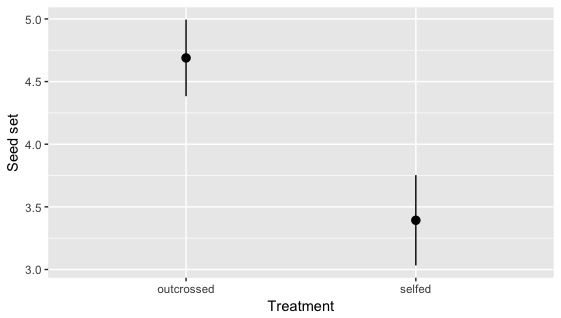
As the native plant industry continues to mature and expand production to include a greater diversity of plants for ecological restoration, researchers and practitioners must gain a deeper understanding of ecological and biological plant traits that will maximize both economic and ecological success for the native plant industry. This study has demonstrated that manipulating the breeding system of Pennsylvania Sedge to facilitate outcrossing may be a promising technique to improve seed yield for this difficult to produce species. Because of the prevalence of inbreeding depression in many native plants species under natural conditions and the growing prevalence of fragmented landscapes that isolate individual populations, growers will increasingly need to explore options for cross-breeding among several populations. Seed gardens that are spatially-oriented to increase pollen transfer between genets may be a useful tool to improve the seed yield in other taxa that are underrepresented in ecological restoration projects. The benefits of continued research in the breeding systems of important restoration plant species would extend beyond viable plant production in the nursery industry. The breeding system is a strong control of population genetics in plant systems. Applied inquiries into the breeding systems of plants used in restoration could provide missing pieces of the puzzle in restoration planning, enabling managers to restore populations with the genetic capacity to persist in the long-term and the potential to evolve and keep pace with the changing conditions of our world.

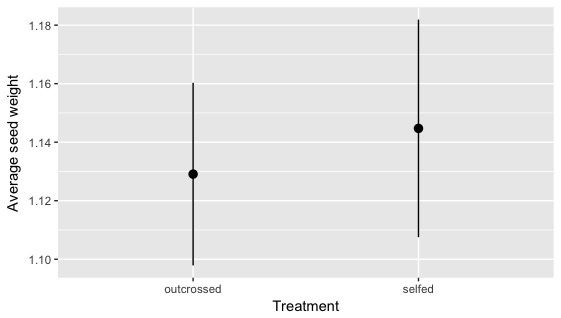
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**Figures:**

**Figure 1:** Mean value and standard error of seed set for *C. pensylvanica inflorescences in 2015-16 in Ann Arbor, MI* under obligate outcrossing and selfing experimental treatments. Seed set was significantly higher in outcrossed flowers than in selfed flowers (P<0.0068)

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**Figure 2:** Mean value and standard error of average seed weight for *C. pensylvanica inflorescences in 2015-16 in Ann Arbor, MI* under obligate outcrossing and selfing experimental treatments. No significant difference in seed weight (P= 0.748) was observed between outcrossing and selfing pollination treatments. ****

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