The Rate and Characterization of Hybridization Between Wild-Type and Cultivated Switchgrass (*Panicum virgatum* L.) for Biofuel Use

A Senior Honors Thesis

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ABSTRACT

The introduction of new and widely planted cultivars for biofuels raises questions about their potential invasiveness compared to their wild-type counterparts. Switchgrass is a native, self-incompatible prairie grass that has been bred for biofuels and may be genetically engineered in the near future. Baseline information is needed about the rate of crop-wild hybridization to assess possible consequences of gene flow. In 2011 our research group established an experimental stand of 106 cultivar "pollen donors" at The Wilds, Cumberland, OH, surrounded by wild "pollen recipients" at distances ranging from 1-100 m away. The donors and recipients were derived from two distinct clones, each carrying two unique alleles at a specific SSR locus. I studied DNA from their seeds to see if they showed evidence of 1) hybridization as expected (due to self-incompatibility), 2) fertilization by unidentified switchgrass sources, or 3) self-fertilization.

After optimizing my methods, I used capillary electrophoresis to genotype 8 F1 progeny from the donor plot, 16 progeny from wild recipients at each of three distances (1 m, 30 m, and 60 m), and 32 progeny from wild recipients growing 100 m away. I found that 100% of the offspring analyzed were heterozygous for the target SSR markers, confirming crop-wild hybridization at distances up to at least 100 m. I also found no evidence of self-fertilization or fertilization by another source of switchgrass. My results suggest that our research group can use seed set instead of costly DNA analyses to measure gene flow at this study site. In addition, the cultivar's ability to pollinate native switchgrass at distances of at least 100 m suggests that distance should carefully be considered before novel biofuel cultivars are planted in a new location or in field trials involving regulated transgenic switchgrass.

INTRODUCTION

Several long-lived perennial biofuel crops are ready to be put to commercial use, but the possible long-term effects of these new crops on the entire ecosystem are not known. Desirable traits for a successful biofuel crop – high yield, increased efficiency, and drought tolerance among others– could increase the competitive ability after the plants have been selectively bred for several generations (Raghu et al 2006, Barney et al 2008). Interest in switchgrass (*Panicum virgatum)* as a bioenergy source began in 1985 when it was consistently observed to have high yields and low nutrient and water requirements (Parrish and Fike 2005). Selective breeding for these traits and the use of non-local genotypes has resulted in lines of cultivated switchgrass distinct from the native wild-type in mid-western grasslands. Little is known about the rate of hybridization between switchgrass cultivars and their wild relatives, or the farthest fertilization distance attainable by the cultivars.

In the past, switchgrass has been used for erosion control, prairie restoration, forage, runoff reduction, landscaping and ornamental purposes, but the demand for alternative sources of fuel has opened up the possibility of using the considerable biomass of switchgrass as a biofuel, whether through direct combustion or conversion to cellulosic ethanol. To produce ethanol, sugars are chemically extracted from collected biomass, which are then fermented into alcohol by certain microbes (Sticklen 2008). Ethanol derived from livestock and human food sources, notably corn and sugarcane, already makes a small but important contribution to the global fuel industry (Balat and Balat 2009). However, perennial prairie grasses such as switchgrass show substantial promise to augment the contribution of biofuels. It is not a source of food, and utilization of

switchgrass could increase availability of those biofuel sources that are. Beside this economic benefit, switchgrass can also grow in a variety of edaphic conditions that cannot be used for crops without nitrogen and other nutritional supplements (Tilman et al 2006). The natural phenotypic variation of switchgrass allows for great flexibility in soil conditions (Sanderson et al 1996), and as development of cultivar strains continue, the range of this prairie grass is likely to expand. The low input required and high rate of return on resource investment indicated by the relatively high calculated net energy balance ratio gives switchgrass and other prairie grasses an advantage in the fuel market (Hill et al 2006, Roach and Meir 2012). With similar or greater energy yield per hectare than corn, the leading source of biomass-derived ethanol in the United States, it is feasible for switchgrass to become a more popular alternative fuel source pending corresponding advancements in the alcohol conversion process (Roach and Meir 2012, Hill et al 2006).

Before widespread cultivation of switchgrass for biofuel use can become a reality, further evaluation of its potential weediness should be undertaken. As mentioned above, cultivar strains have been selectively bred for traits advantageous to an efficient biofuel source. This includes increased biomass, drought tolerance, heightened nutrient acquisition efficiency, and other qualities that may increase the competitive ability of switchgrass compared to its wild-type relatives and other native prairie grasses (Kwit & Steward 2011). These traits are also being assessed for transgenic modifications and examined in current field trials, conducted through various universities (Albert Kausch, University of Rhode Island; Stephen Dellaporta, Yale University; Rongda Qu, North Carolina State University; and Neal Stewert, University of Tennessee) and companies (Mendel Technology, Ceres, Endospace). The enhanced properties of cultivated or transgenic

switchgrass and repetitive planting and propagation that accompanies field trials and commercial farming comes with possible risks: often it is not until there is a strong established presence that a plant will naturalize to the new environment and possibly become weedy (Mack 2000). The invasive properties of improved switchgrass have yet to be fully characterized, though in some preliminary experiments cultivars were found to have higher survival rates and greater biomass production when they were fertilized equally to remnant prairie populations (Hopkins et al. 1995, Casler 2005). Although methods of transgenic bioconfinement exist, such as cytoplasmic male sterility or lack of seed production caused by genetic mutations, all of the known methods have limitations in their effectiveness and many are not yet available to the biofuel movement (Kausch 2010). Therefore there is significant risk to introducing cultivated and transgenic switchgrass to native and non-native habitats where it could flourish in the United States.

The risk of using cultivated and transgenic switchgrass as biofuels would be significantly lower if we could ignore the potential for gene flow. Depending on the species, problematic introgression of novel genes (e.g., certain transgenes) can occur between genetically related species, particularly wild relatives, and these introduced genes may persist in that new environment with unknown consequences (Stewart et al 2003). Current cultivars are still closely related to wild-type switchgrass, so their similar gene pool is less likely to "contaminate" native populations (Casler et al 2007). However, the hybridization and possible gene flow from cultivar to wild-type should not be ignored, as too little information is known about the effects of introducing anthropogenically improved switchgrass to a disturbed or vulnerable ecosystem, where it has a greater chance to become weedy and invasive, and change the biodiversity of that ecosystem.

The study of hybridization between available cultivars and wild-type switchgrass can lead to greater insights on the limitations of large-scale cultivation, should switchgrass realize its full potential as a biofuel. The dispersal of selected genes into the wild population is often seen through pollen-mediated fertilization when it results in fertile hybrids (Stewart et al 2003), and seed-mediated dispersal is a concern as well. Using molecular markers as confirmation, we attempted to determine if switchgrass fertilization by a donor source was possible at distances up to 100 m. Additionally, my individual aim was to assess the relative rate of self-fertilization, hybridization, and fertilization by switchgrass pollen unaccounted for in the experimental set up. This baseline knowledge will aid in risk assessment for cultivar and transgenic switchgrass introduction to areas that may contain their wild-type counterparts.

METHODS

Study Site Description

The field plot where the study was conducted is located at The Wilds, a 9,154-acre wildlife conservation center in Cumberland, OH (CZA 2012). Set up by Hsiaochi Chang in 2011 on a USDA-funded project in the lab of Dr. Allison Snow, the experiment illustrates the pollen movement and gene flow between cultivated (donor) and wild-type (recipient) switchgrass. As shown in Figure 1, we used a pair of clonally propagated individuals from three-year-old switchgrass used in a previous garden study, one of which was a native variety taken from the restored prairie at the Ohio State University Marion campus and the other a Summer cultivar. Because ramets from these clones would be genetically identical, they are self-incompatible and it could be ideally assumed that fertilization would occur

only between genetically distinct individuals (Martínez-Reyna and Vogel 2002). Part of my objective was to ascertain the validity of this assumption. Before their selection, it was ensured that they had the same ploidy level (4) to allow for hybridization (Martínez-Reyna and Vogel 2002) and they also had unique Simple Sequences Repeat (SSR, M19) DNA markers (developed by Tobias et al 2006) at the selected locus to later genotype the progeny.

The 106 cultivar donor plants were arranged in an octagonal shape made up of 12 rows, with each row containing up to 11 clones. Along cardinal and sub-cardinal directions, wild-type sentinel triplets were placed at distances of 1m, 30m, 60m, and 100m. In the design depicted in Figure 2, each transect represents a group of 3 wild-type recipients at each specified distance.

DNA Analyses

Each seed collected was considered an individual fertilization event, and the selected samples are summarized in Table 1. The seeds were soaked, heated at 35°C to release them from dormancy, and raised in the greenhouse for 3-5 weeks before DNA extraction was performed on leaf samples using the paper chromatography protocol on Whatman paper (Adugna et al 2011). Samples with squashed leaf tissue were washed twice each with FTA reagent and TE (0.1 M), then soaked in TE (1M) at 95°C for five minutes to fully extract the DNA into the solution. Control samples from the parental plants were completed alongside their progeny.

The extracted DNA in TE solution was subjected to Polymerase Chain Reaction (PCR), optimized for the primers chosen to amplify the section of DNA containing the

selected unique SSR markers after a refined marker selection. In the original marker screen, several options were identified and one was selected out of 32 expressed sequence tags (ESTs), or SSRs (previously developed at USDA-ARS , Tobias et al. 2006) to differentiate between the donor and recipient clones. During my initial work, I found the selected marker could not reliably distinguish hybrids due to the heterozygosity of one of the parental plants at that locus. I then analyzed the other possible options identified in the original marker screen, and SSR 5008_B05 was determined to be truly unique to each clonally propagated switchgrass plant. The accompanying primer had a forward (5'-3') sequence of GCTGATTGCTCAATCCTGCT and a reverse (3'-5') sequence of ACCTCCATTGGTCACAACACA. The PCR mixture per reaction contains 1 μL mixed forward and reverse primers (labeled with FAM fluorescent dye), 5 μL HotStart PCR Premix, 2 μL doubly distilled water, and 1 μL template DNA. The PCR program starts with an activation time of 15 minutes at 95°C, followed by a 40-cycle process consisting of denaturation for 30 seconds at 95°C, annealing for 90 seconds at 55°C, and extension for 60 seconds at 72°C. A final extension time of 30 minutes at 72°C completes the reaction, and products are stored at 4°C without light interference.

To genotype the progeny and the parentals, capillary electrophoresis was completed using the ABI Prism 3100 genetic analyzer (Applied Biosystems). Each of the 96 PCR products was diluted with 20 μL doubly distilled water and combined with 0.5 μL GeneScan 350 ROX internal size standard and 15 μL formamide to be denatured at 95°C (5 min), cooled on ice, and sent for analysis. To determine hybridization, the presence of one allele fragment from each parental source (190 or 197 bp from the donor, and 195 or 202 bp

from the recipient) was confirmed at the selected locus using GeneMapper software (version 4.0, Applied Biosystems) to detect peaks (shown in Figure 3A-C).

RESULTS

Allelic scoring revealed no evidence of self-fertilization or fertilization by another switchgrass source. The nearest known switchgrass source was about 5 km away, but the possibility existed of an unknown or known endemic plant fertilizing recipients, particularly at distances farther away from the donor plot. Twice as many progeny were sampled from 100 m to possibly find any evidence of fertilization by alternative switchgrass plants. 100% of the recipient and donor progeny were found to be hybrids, including those at a distance of 100m (Table 2). Capillary electrophoresis failed on 2 of the samples, resulting in a final sample size of 86 progeny (8 donor and 78 recipient), all of which contained one allele from each parental switchgrass plant (Figures 3A-C).

DISCUSSION

Pollen-mediated gene flow

The 100% hybridization rate confirms that our research group can use seed set instead of costly DNA analysis to measure gene flow at this site. X-ray analysis is much more time and cost efficient, and can differentiate between a fertilized seed and non-fertilized seeds produced. Furthermore, we have realized that to ensure homozygosity of the parents in the initial marker screen and selection, more samples should be taken and analyzed. I searched for evidence of self-fertilization or alternative pollen sources in the field but since no evidence of either was found, fertilized seeds can be assumed to be

hybrids between the cultivars and wild-type. The high frequency of hybridization additionally demonstrates that the flowering times of cultivars and wild-type overlap, and hybridization occurred at each distance tested. The inverse relationship between fraction of seed set and distance from the donor plot is displayed in Figure 4 (H. Chang, unpublished data).

The cultivar's ability to pollinate native switchgrass at distances of at least 100 m means that isolation distance should be carefully considered before novel biofuel cultivars are planted in a new location or regulated transgenic switchgrass field trials are approved. In a similar study, evidence of gene flow between transgenic creeping bentgrass, a wind-pollinated, perennial crop, and its wild-type relatives was found at maximal distances of 21 km (Watrud et al 2004). Further studies, perhaps up to several kilometers in radius, should be considered to ascertain true switchgrass pollen viability, or its functional ability to fertilize another plant (Dafni and Firmage 1999). Another gene flow study on the wind-pollinated grass *Festuca pratensis* used two populations homozygous for different allozymes at the *Gpi-2* locus, and could determine heterozygosity by the presence of both allozymes in the progeny. However, at larger distances, they were unable to distinguish donor pollen from feral pollen, owing to the small amount of polymorphism (4) of the allozyme (Rognli et al 2000). The use of highly polymorphic SSR markers has proven to be more accurate and reliable than other methods, and could be utilized in these further studies.

Wind direction should also be taken into account, as that appeared to play a role in the recipient fertilization rate as distance from the donor plot increased. In this location for example, many more fertilized seeds were observed in the south and east directions than

the north and west (H. Chang, unpublished data). Density and size of the donor plot is extremely influential on the level of gene flow (Rognli et al 2000), so our relatively small-scale experiment is more relevant to the immediate demand for field trials involving transgenic switchgrass than the resulting large scale planting if switchgrass becomes a common source of biofuel. The observed fertilization of plants up to 100 m from the small donor source of pollen could be a cause for concern if any transgenic switchgrass is planted in an area with feral populations nearby.

Broader Implications

Private companies and federal agencies have invested large amounts of time, funds, and resources to the development of switchgrass to be used as a source of bioethanol. Despite the pressing desire for lower carbon emissions and alternative fuel sources, enthusiasm for new cultivars and GM lines of switchgrass must be tempered with caution. The need for more studies like this one, meant to evaluate the risk of widely planting new biofuel sources, are necessary to prevent the introduction of an invasive or weedy species to an area.

Many examples exist of well-meaning government or individual sponsored introduction of a non-native species to a new environment that has drastically altered the entire ecosystem. One such story of devastation is that of kudzu, an Asian, semi-woody, perennial vine that is highly invasive to the Southeastern United States. Originally employed by a few individuals to provide shade over supported trellises, by the 1930's the U.S. government had chosen kudzu as a method for controlling the massive soil erosion in the Southeast, a problem compounded by the Great Depression. The federally-led

promotional campaign for kudzu resulted in a well-established and highly invasive alien species due to the purposeful widespread planting of the vine (Blaustein 2001). It continues to wreak havoc on the area's natural ecosystems and agricultural lands, stifling biodiversity and other native flora. This is an extreme case; however, our study and others like it are meant to prevent such a disaster from occurring again, whether by invasive properties of the plant itself or gene flow into surrounding populations.

There are many unanswered questions regarding the consequences of using cultivars or GM switchgrass on a large scale for biofuel production. Before we know more, it cannot be assumed that the benefits to extensive switchgrass propagation in preparation for biomass harvesting outweigh the potential harmful effects of gene flow or introduction of prairie grasses to different habitats. A sample size of one or two environmental sites to test the possibility of this is not enough – no two ecosystems have the same dynamic forces at work and they often respond to changes in unpredictable ways. Invasive species and climate change are two of the most prevalent forms of ecosystem disturbance now in the 21st century, and sometimes ecosystem disturbance caused by climate change fosters the prosperity of invasive species, further disrupting the system (Bradley et al 2009). Both issues, climate change and invasiveness of non-native species, increasingly threaten biodiversity and are becoming difficult to ignore. The ability of humans to alter the very genome of other species should not be handled lightly, for evolutionary processes took millions of years to develop the organisms we see today. Huge advantages can be gained from selective cultivation and genetic modification of biofuel feedstocks but with every technical advance, cautionary steps must be taken to ensure we will not be worse off than we were before.

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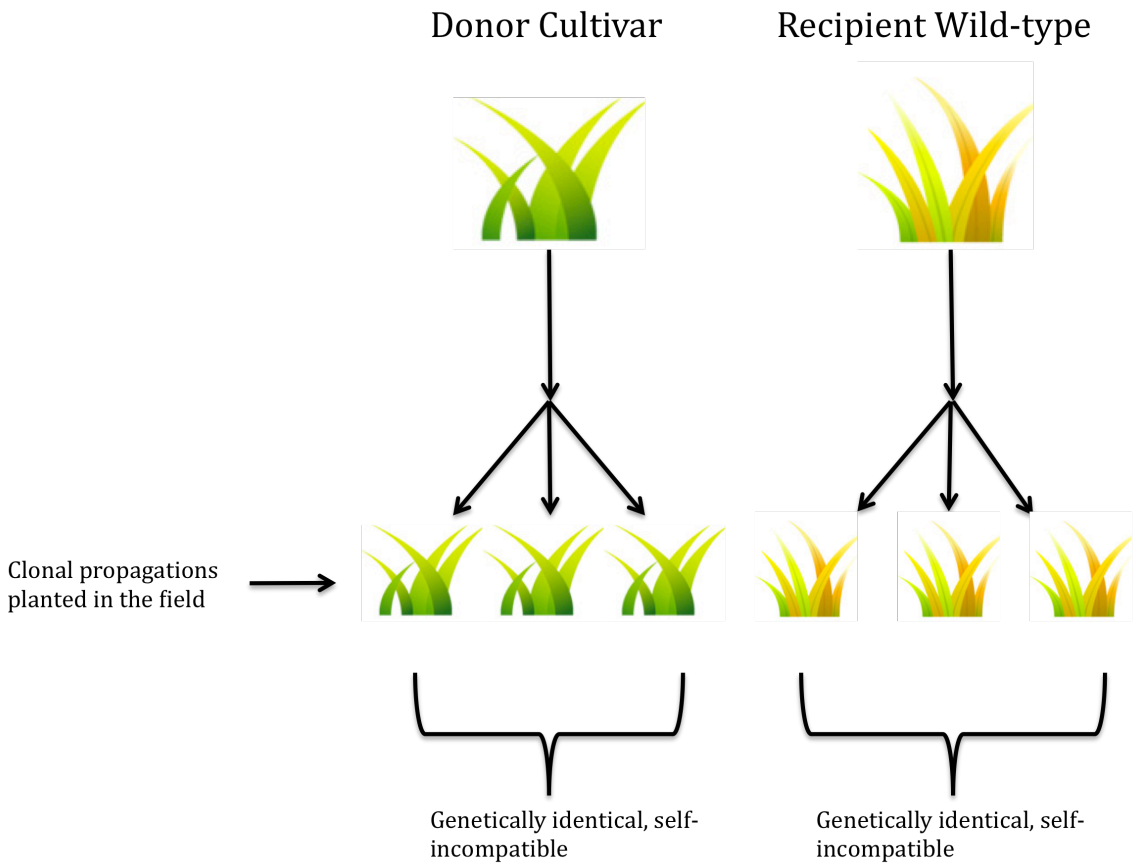


Figure 1. Schematic for clonal propagation. This creates genetically identical plants of each variety so that the donors cannot fertilize other donors, and the recipients cannot fertilize other recipients (concept of self-incompatibility).

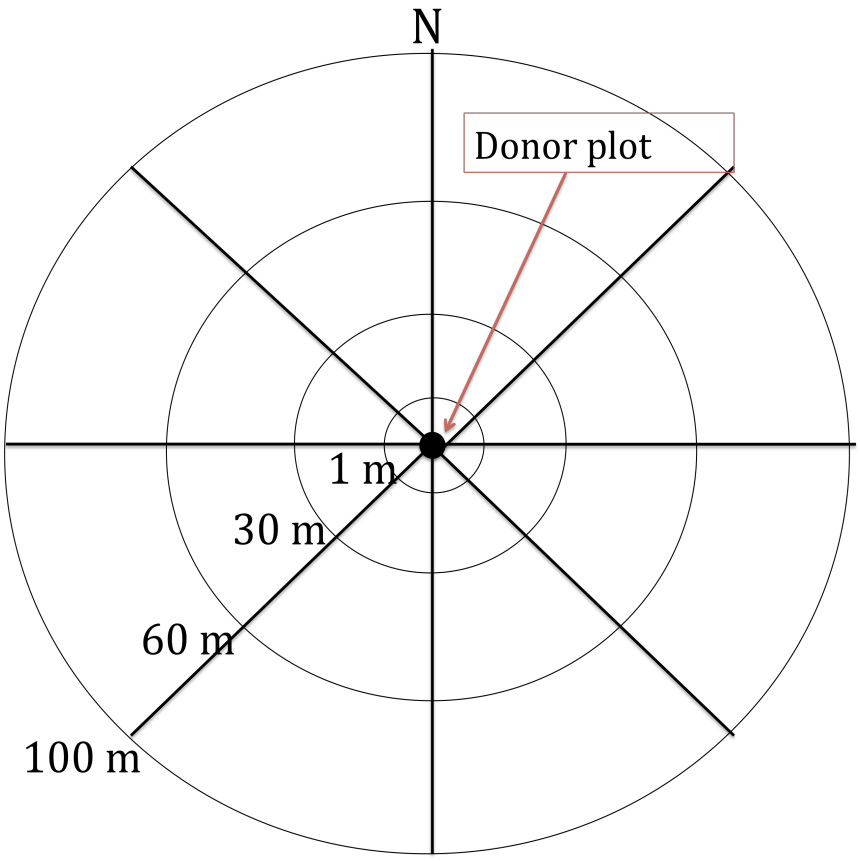


Figure 2. Experimental Design – The Wilds, Cumberland, OH
The donor plot at the center consists of 106 cultivar "pollen donor" clones surrounded by small groups of wild "pollen recipients" radiating from the center plot. There were 8 examined directions with 3 pollen recipients at 1, 30, 60, and 100 m from the source, represented by each transect.

	Donor	1 m	30 m	60 m	100 m
Number of Samples	8	16	16	16	32

Table 1. At each distance, each cardinal and subcardinal direction is represented (approximately 2 seeds from each at 1m, 30m, 60m; 4 seeds from each at 100m).

	Self-Fertilization	Hybridization	Fertilized by other source
Donors	0% (0/8)	100% (8/8)	0% (0/8)
Recipients	0% (0/78)	100% (78/78)	0% (0/78)

Table 2. The results of capillary electrophoresis revealed no evidence of self-fertilization or fertilization by another source of switchgrass. All of the progeny tested were found to be hybrids.

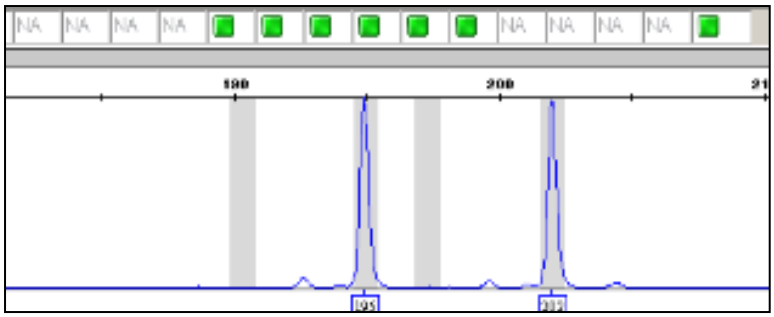


Figure 3A. Parental Genotype – Recipient Wild-type
Each peak represents an allele unique to the cloned wild-type switchgrass used in the experiment (195 and 202 bp) at the selected SSR locus.

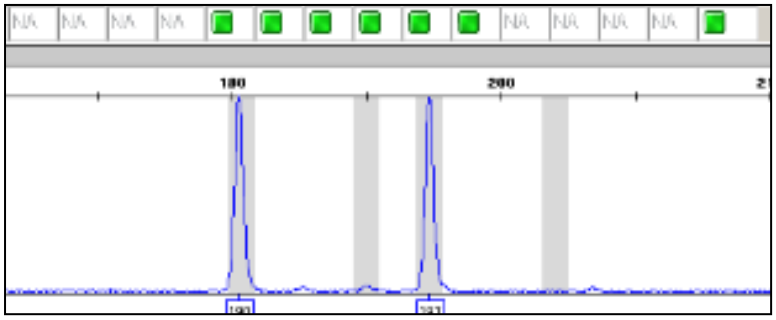
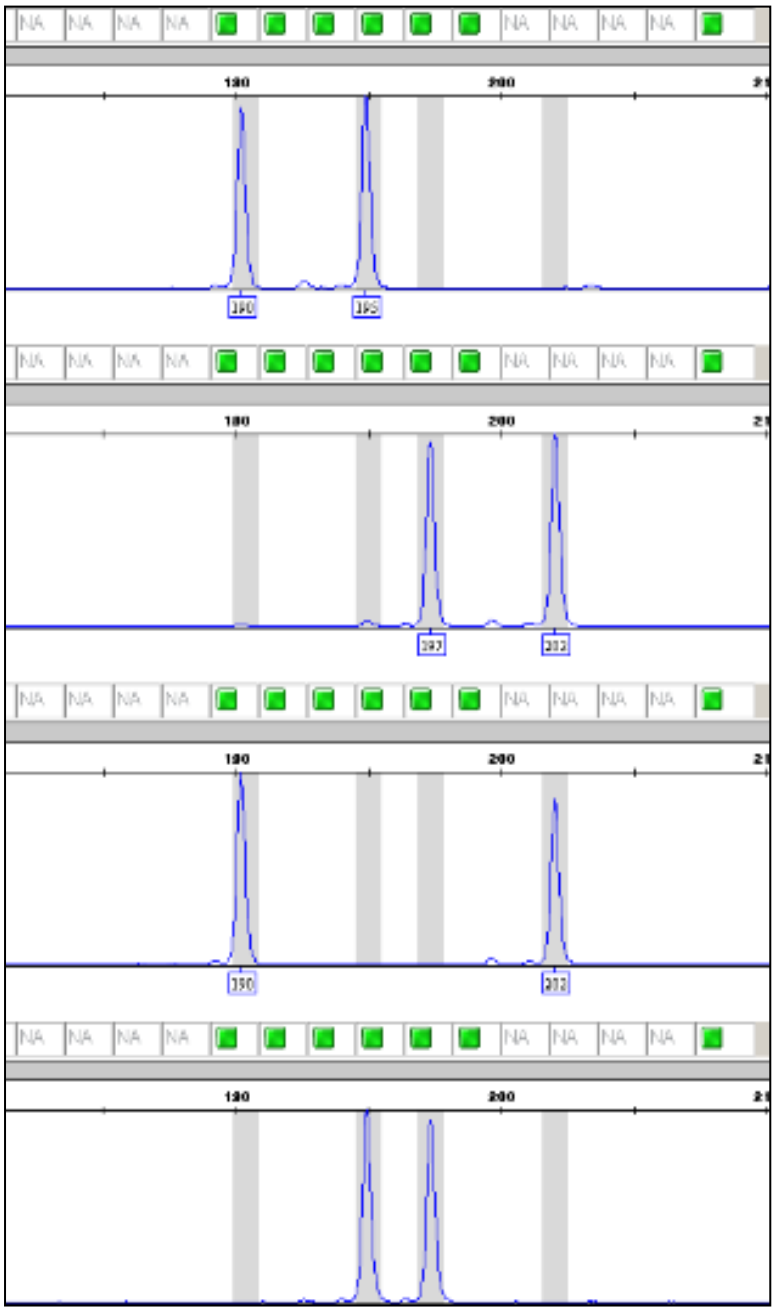


Figure 3B. Parental Geneotype – Cultivated Donor
Each peak represents an allele unique to the cloned cultivars (190 and 197 bp) at the same SSR locus. The presence of one of these alleles in the progeny genotypes indicates fertilization by the donor plants.



Percent of Progeny

22.6%

20.2%

22.6%

34.5%

Figure 3C. Offspring Genotypes – 100% Hybridization
The four genotypes shown here are the four variations of genotypes that would result from hybridization of the two parental switchgrass plants. Each of the 86 offspring analyzed had one of these four genotypes, serving as molecular confirmation of hybridization. A Chi squared test revealed no statistically significant deviation from expected results for a Mendelian inheritance pattern and independent assortment of alleles (X^2calc = 6.08, n=4, p>0.10).

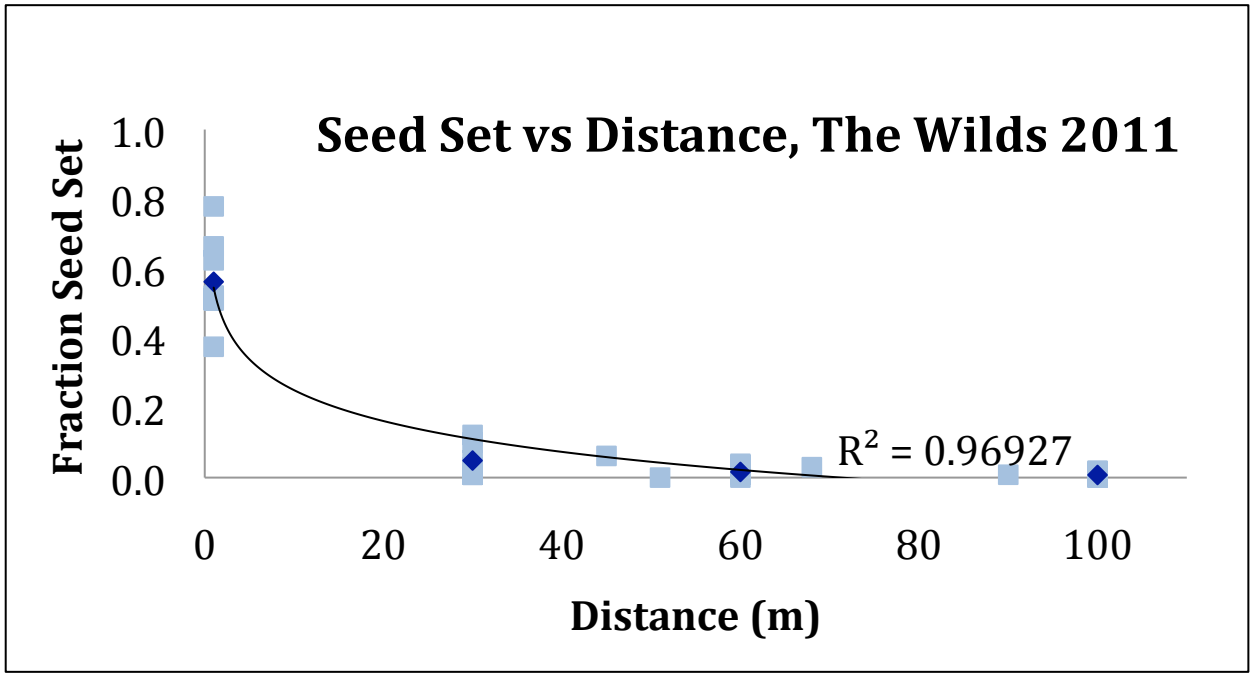


Figure 4. (Hsiaochi Chang, unpublished data) The inverse relationship between the fraction of seed set out of the total amount of florets produced and the distance from the donor source is clearly shown by this figure. As distance increases, the fraction seed set decreases to almost nothing at distances of 100 m. However, the presence of viable seed this far from the donor plot could be a cause for concern when considering potential pollen dispersal of switchgrass.