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Research paper

Extent of pollen-mediated gene flow and seed longevity in switchgrass (*Panicum virgatum* L.): Implications for biosafety procedures

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ABSTRACT

New switchgrass (*Panicum virgatum* L.) bioenergy cultivars are being bred through genetic engineering; however, baseline information is urgently needed to establish guidelines for small-scale field trials prior to commercialization. In this study, we documented the pattern of pollen-mediated gene flow and the extent of seed longevity in field experiments. To mimic crop-to-wild, pollen-mediated gene flow, we planted wild recipient switchgrass ramets at various distances away from cultivar donor ramets at two sites in Ohio. Percent hybridization at each distance was estimated from seed set on recipient ramets, which were self-incompatible clones. The pattern of gene flow was best described by negative exponential models, and the minimum isolation distance for a 0.01% gene flow threshold was predicted to be 69 m and 109 m away from the pollen source at the two sites. To investigate seed longevity, we buried seeds of six cultivars and ten wild biotypes in Ohio and Iowa in 2011. A subset of the seeds were exhumed, germinated, and tested for dormancy over three years. Cultivars lost seed viability and dormancy significantly sooner than wild biotypes at both locations in the first year, and most biotypes lost dormancy by the second year. Cultivar seeds buried in the cooler, drier Iowa site had an overall greater longevity than those buried in Ohio. Our findings suggest that substantial amounts of pollen-mediated gene flow could occur in the immediate vicinity of switchgrass pollen sources, and current switchgrass cultivars are unlikely to persist in the seed bank for more than three years.

1. Introduction

Lignocellulosic "second-generation (2G)" bioenergy crops, for instance, switchgrass (*Panicum virgatum* L.) and miscanthus (*Miscanthus* spp.), have attracted much attention because they (a) utilize the more efficient C_4 photosynthetic pathway, (b) have a perennial growth habit, extensive root systems, and abundant, harvestable biomass, and (c) tolerate poor growing conditions by having relatively high nutrient and water use efficiency [1–3]. These traits combined with other considerations, such as providing ecosystem service, offer advantages while minimizing competition with major food crop production [4–6].

Switchgrass has become a potential bioenergy crop in the U.S. in part because of its native origin, minimal pest and disease problems, and great adaptability to a broad range of environments. It is a perennial C_4 grass that was commonly found throughout the U.S. east of the Rocky Mountains before European settlement [7]. While unique wild switchgrass populations are still present and scattered across the landscape [8], domesticated switchgrass has been in demand as a multipurpose crop across the U.S. Since the 1980s, switchgrass has been planted for animal feedstock, wildlife habitat, and soil restoration [9–12]. With the need to develop renewable energy sources, it has been studied as a model bioenergy species in the U.S. during the past three decades, alongside multimillion dollar investments in breeding and cultivar development [13–15].

In the last decade, genetic engineering has been used to expedite switchgrass breeding programs [16,17]. For instance, by overexpressing a transcription factor gene, Baxter et al. [18] created a switchgrass line with lower lignin content, resulting in significantly higher biofuel and biomass production (32% and 63%, respectively) compared to the non-transgenic control group. In another study, a sucrose synthase gene was overexpressed, which produced greater plant height, tiller number, and biomass relative to control plants [19]. Insertion and overexpression of a maize microRNA into switchgrass improved starch content and digestibility, and it also prevented switchgrass from flowering, therefore avoiding potential gene flow through pollen [20].

The development of transgenic switchgrass for bioenergy has

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generated discussion of the potential evolutionary consequences if transgenes are introduced into wild populations through gene flow. For instance, prolific aboveground production and fast growth are commonly found in weeds [21,22]. If new switchgrass cultivars possess significant fitness advantages and escape from cultivation, they could establish and possibly change the dynamics of local ecosystems in which they are planted, or to which they are dispersed [22]. In addition, hybridization between advanced cultivars and wild relatives could potentially replace local gene pools through pollen, seeds, or vegetative propagules [23–26]. Furthermore, if the introduced transgenes provide wild relatives with more advantageous traits, weed management could become more difficult and costly [24]. As of October 2017, the U.S. Department of Agriculture (USDA) has granted permission for thirty-five controlled open air field release involving transgenic switchgrass [27]. However, the extent of gene flow in switchgrass under field conditions, whether through pollen or seed, has not been well characterized. Without such information, efforts to minimize potential ecological impacts of transgenes are compromised. The same information is also urgently needed for commercialization of future transgenic switchgrass and conservation of wild switchgrass populations [23].

Despite interest in breeding 2G bioenergy crops, existing gene flow studies have mainly focused on food crops, and our understanding of gene flow in emerging bioenergy crops remains limited [21,23]. Moreover, while multiple switchgrass cultivars have been developed for forage and energy, ecological characteristics of new cultivars have not been well characterized. For example, the extent and variability of pollen-mediated gene flow in switchgrass under field conditions have only been recently described in the literature [28]. The pattern of pollen-mediated gene flow is dictated by numerous factors, for instance, climatic conditions, pollination biology, and size and proximity of source and recipient populations; therefore, patterns of gene flow are expected to vary in different landscapes [29–31]. Seed dispersal also contributes to gene flow. Switchgrass seeds are lightweight and can easily disperse from planted fields through wind, water movement, or during harvest and transportation. Furthermore, dispersed seeds emerging in the subsequent year, i.e., as volunteers, could act as a secondary source of gene flow [32]. Nevertheless, we are not aware of a systematic comparison of seed persistence between domesticated and wild switchgrass.

As 2G bioenergy crops go forward, the USDA has demanded more thorough ecological studies for making regulatory decisions [22,23,28,29,33]. Specifically, data are needed to characterize the minimum isolation distance between switchgrass carrying novel transgenes and sexually compatible relatives, and the longevity of these seeds when left in the soil. One of the goals of this study was to measure the frequencies of pollen-mediated gene flow in switchgrass from a small donor source to recipient plants at different distances from the source, inferring the characteristic gene-flow radius using the frequency of crop-wild hybridization versus distance in two locations. In addition, this study compared seed viability and dormancy in cultivated and wild switchgrass in two common garden experiments over the span of three years. To our knowledge, this study is the first to document pollen-mediated gene flow in switchgrass under field conditions and to compare seed longevity in switchgrass cultivars and wild biotypes over a period of several years. This study aimed to provide guidance for future regulation of transgenic switchgrass developed for bioenergy, especially for field trials prior to deregulation and commercialization. It also aimed to shed light on the long-term conservation of remnant switchgrass populations, which often occur near agricultural land.

2. Materials and methods

2.1. Pollen-mediated gene flow

To track pollen movement from cultivated to wild populations, we recorded the frequencies of crop-wild hybridization in experimental arrays at two sites in Ohio (Fig. 1). We used clonally propagated ramets from one pair of three-year-old, non-transgenic switchgrass clones at each site, with cultivar ramets as the pollen donors and wild ramets as the pollen recipients. Because switchgrass is self-incompatible and the ramets were genetically identical [34], they could only hybridize with genetically distinct individuals present in the field. Therefore, any seeds present on the recipient plants should be crop-wild hybrids, provided that no other pollen sources occur nearby. Note that switchgrass is generally believed to be an obligate outcrossing species; however, it is possible that an isolated individual could self in rare circumstances. Hence, we included molecular analysis to check for paternity status of the putative hybrid seeds using microsatellite DNA markers.

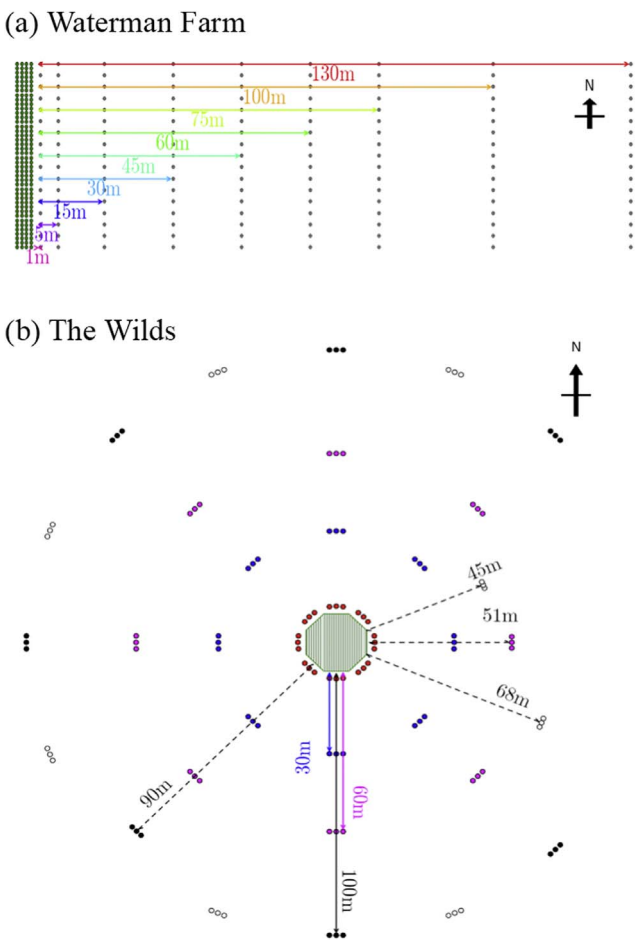

Fig. 1. Layouts of the experimental plots at (a) Waterman Farm and (b) The Wilds. At Waterman Farm, donors (cultivar ramets) were planted in four rows (green dots), and the recipients (wild ramets) were planted to the east side of the donors (gray dots). At The Wilds, donors were planted in the center (green octagon), and recipients were planted in triplets. Trees were present on the east side; therefore, planting of recipients was restricted. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.1.1. Selection of pollen donors and recipients

First, we selected crop donors and wild recipients with the same ploidy level, because hybridization between different ploidy levels rarely occurs in switchgrass [34–36]. We selected two wild clones that were originally collected from a restored prairie in Marion, Ohio, and maintained as accessions in a common garden study [35]. We determined their ploidy levels using a *BD™* LSR II flow cytometer at the University Cell Analysis and Sorting Core at the Ohio State University (OSU), Columbus, OH [37]. Following the protocol in Galbraith et al. [38], the genome size of each sample was estimated based on a minimum of 3000 nuclei count using *BD™ FACSDiva*, and ploidy was

inferred by comparing the mean peak value of the sample with that of internal standards (a 4x or a 8x switchgrass cultivar sample with known genome size). The two wild recipient clones were tetraploid (4x); hence, we used clones of a tetraploid (4x) cultivar, known as "Summer", for additional screening in the next step. Summer is a common cultivar that is used for range and pasture. It originated from Nebraska and was bred for early season vigor, leafiness, and rust resistance [39].

Second, to determine whether seeds on recipient plants were the expected crop-wild hybrids, we screened for distinctive microsatellite DNA markers in the two wild clones and candidate Summer clones following the protocol in Mutegi et al. [25]. We identified alleles unique to the two wild and two Summer clones using marker 5008_B05 in Tobias et al. [40]. The first parental pair (later planted at Waterman Farm) had two copies of an allele with 193bp (from the cultivar parent) and alleles with 199bp and 202bp (from the wild parent). The second parental pair (later planted at The Wilds) had alleles with 185bp and 193bp (from the cultivar parent) and alleles with 190bp and 199bp (from the wild parents). Each of the four clones was divided into 100–200 ramets on May 20, 2011, and allowed to grow in a greenhouse until ready for transplanting to the field sites.

2.1.2. Hybridization rate and confirmation of paternity

We recorded the flowering of cultivar and wild ramets at each site, and tagged panicles that were at the onset of flowering with color labels each week. To estimate hybridization rate, we referenced the color labels and harvested florets from recipient panicles that bloomed during the peak flowering overlap of donors and recipients (i.e., five weeks). Hybridization rate was defined as the ratio of the number of fully developed seeds divided by the number of florets that were counted. To calculate hybridization rate, florets from a random subset of panicles at each recipient distance were counted, stripped, and X-rayed at the Ornamental Plant Germplasm Center, Columbus, OH (Fig. 2). A matured seed appeared white under X-ray, whereas unpollinated (thus hollow) floret appeared gray. Panicles were harvested from Waterman Farm on October 28, 2011, and from The Wilds on October 23, 2011. At Waterman Farm, due to small plant size (Appendix A), panicles were pooled from each recipient distance, for a total of nine data points, and ~300 florets from each distance were screened. At The Wilds, we calculated hybridization rate at each recipient position by averaging measurements from each recipient triplet at a given recipient distance. We screened all of the florets (approximately 4500–8000 florets per distance) due to the smaller numbers of panicles produced (Table 1). To detect pollen contamination from unknown pollen sources or as a result of selfing, we obtained seeds from harvested florets and screened a subset of the seeds for each recipient distance using the microsatellite marker described earlier (sample sizes for the DNA analysis are shown in Table 1).

Table 1
Screening of putative hybrid seeds from two Ohio experimental sites (Waterman Farm and The Wilds). A subset of florets was subject to X-ray to calculate fraction of hybridization at the designated recipient distance. To confirm hybrid status, additional seeds were screened for alleles unique to the paternal cultivar donors using DNA microsatellite markers.

Distance (m)	X-Ray Imaging			Microsatellite DNA Marker Screening = p
	No. Florets Screened	No. Seeds Found	Fraction Hybridization	No. Seed Screened (No. True Hybrid) = p
Waterman Farm				
1	493	357	0.72	8 (8) = 100%
5	300	120	0.40	8 (8) = 100%
15	300	38	0.13	8 (8) = 100%
30	300	18	0.06	8 (8) = 100%
45	300	2	0.01	8 (8) = 100%
60	300	3	0.01	4 (4) = 100%
75	300	3	0.01	4 (4) = 100%
100	307	0	0	-
130	300	13	0.04	8 (8) = 100%
The Wilds				
1	4831	2710	0.56	16 (16) = 100%
30	6158	308	0.05	16 (16) = 100%
60	4486	77	0.02	16 (16) = 100%
100	8087	704	0.01	32 (32) = 100%

2.1.3. Sites and experimental design

One of the experimental plots was established at the Waterman Agriculture and Natural Resources Laboratory Complex, Columbus, OH (30.0169°, −83.0409°). Waterman Farm is an experimental farm with fertile soil and a flat landscape. One of the nearest known switchgrass sources that could have flowered during the same period was a small common garden plot, consisting of 100 tetraploid (4x) switchgrass individuals 0.7 km to the southeast of our plot. Another possible pollen source was a small planting of three ornamental switchgrass clones 0.6 km to the southwest of the plot (ploidy unknown).

The layout of the plot at Waterman Farm was a rectangular design (Fig. 1a) with donors and recipients planted in parallel rows. The donors were planted 0.3 m apart in four rows, with 41 ramets per row, hence a total of 164 ramets. Because the prevailing wind came from the west, all of the recipients were planted on the east side of the donor source in single rows at 1 m, 5 m, 15 m, 30 m, 45 m, 60 m, 75 m, 100 m, and 130 m away, and each recipient row included 17 ramets that were 1 m apart. All of the plants were transplanted in June 2011. Space between the recipient rows was tilled and planted with soybean right after the establishment of the plot.

Another experimental plot was established at The Wilds, a private, non-profit conservation center that covers nearly 10,000 acres of

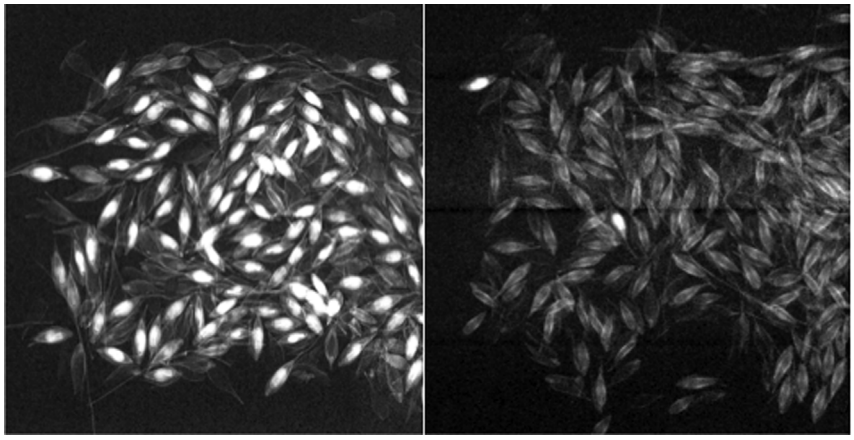

Fig. 2. X-ray image examples of recipient florets collected at 1 m (left) and 100 m (right) from donors at The Wilds. Mature seeds appeared white under X-ray, whereas empty florets appeared gray.

reclaimed mine land in Cumberland, OH. Here, the experimental plot sat on a hill in a grassland area where fescues (*Festuca* spp.), bluegrass (*Poa* spp.), and brome (*Bromus* spp.) were the dominant species (39.8505°, −81.7467°). The plot was more than 3 km away from other known small plantings of switchgrass. Donors were arranged in an octagonal fashion with 12 rows, with each row consisting of 5–11 ramets, for a total of 106 ramets planted 0.3 m apart (Fig. 1b). In existing vegetation, recipients were planted in groups of three to increase pollen interception at each position at 1 m, 30 m, 60 m, and 100 m away from the donors along all cardinal and some sub-cardinal directions, depending on the terrain boundary of each direction. Additional recipient triplets were planted midway between the eight directions at 100 m to increase sampling efforts at this distance. Each recipient triplet position served as one data point, for a total of 39 data points. The donors and recipients were transplanted in June 2011.

2.1.4. Statistical analysis

Several pollen dispersal curves have been reported in other species in the literature, and we fit the most commonly described non-linear regression models, i.e., logarithmic, exponential, and power, to the average hybridization rate as a function of distance in R [41]. Parameters of the models were determined by the common non-linear least square regression method. We considered the model with the highest coefficient of determination (R^2) to be the best model. In addition, we calculated the Akaike information criterion (AIC) of each model for comparison. Using the best model from each site, we quantified the minimal isolation distance at the thresholds of 1%, 0.1% and 0.01% for gene flow.

2.2. Seed longevity

To test for the extent of seed longevity and the effects of "seed types (cultivar *vs*. wild)" and "biotypes (different cultivar varieties, or different wild populations)" within each seed type, we buried sixteen biotypes — six cultivars and ten wild biotypes — and examined their viability and dormancy for three years (Table 2). Four of the cultivars are commercially available and grown across a wide geographic range in the U.S. — Kanlow (KL), Cave-in-Rock (CIR), Sunburst (SB), and Nebraska28 (NE28). We purchased these cultivar seeds from Millborn Seeds Inc., SD, in 2010. We also obtained seeds of two advanced cultivars (ADV1 and ADV2) from Dr. Kenneth Vogel of the USDA-ARS, University of Nebraska in the same year. The commercial cultivars were originally bred for feedstock and soil conservation, whereas the advanced cultivars were selected for biomass production [42,43] (H. Chang and A. Snow, personal communication with K. Vogel and R. Mitchell). The cultivars were harvested in the autumn of 2010, whereas the advanced cultivars were harvested in the autumn of 2007 and had been stored under appropriate conditions for maintaining viability at USDA-ARS. In addition to cultivars, we obtained wild switchgrass seeds from natural areas in Ohio (OH), Illinois (IL), and Iowa (IA) during September and October 2010. These populations are considered as remnant prairies by local environmental specialists [37]. Seeds were stored at room temperature (25 °C) prior to burial, and they were approximately six month old at the time of burial.

2.2.1. Sites and experimental design

We carried out the burial experiment at two locations — Waterman Agriculture and Natural Resources Laboratory in Columbus, OH (30.0169°, −83.0409°, silt loam soil), and Sorenson Research Farm in Ames, IA (42.0125°, −93.7408°, clarion loam soil). To test for the effects of burial location on seed longevity, we buried all cultivar biotypes at both locations. To investigate the differences of seed longevity among wild biotypes, we buried wild seeds in the general regions where they were collected to allow for exposure to local soil — OH and IL wild seeds (six biotypes) were buried in Columbus, OH, whereas IA wild seeds (four biotypes) were buried in Ames, IA (Table 2). Seeds were buried on February 19 and March 1, 2011, in OH and IA, respectively, when the ground became workable and the seeds were still subject to vernalization.

Fifteen hundred seeds of each biotype (50 seeds per replicate for a total of 30 replicates) were buried 20 cm under the soil surface, and 10 of the replicates were excavated after each burial period. Using 200 μm woven nylon mesh, we made 30 seed strips for each site following instructions modified from Mercer et al. [44]. Every strip had numerous packets (~0.5 cm × 2.2 cm), in which 50 seeds of a biotype and a color-coded plastic stake were enclosed. Each seed strip (or replicate) was considered as a "block" where biotypes were randomized within. Each of the seed strips buried in OH contained twelve packets (for six cultivated and six wild biotypes), whereas those buried in IA each had ten packets (for six cultivated and four wild biotypes). The strips were laid flat in a trench to ensure contact with soil and buried in three batches (ten strips per batch) about 20 cm apart and at 20 cm depth to resemble a common depth after tilling.

After seven to ten weeks of burial, the first batch of seed strips (ten replicates) was exhumed on April 7 (OH) and May 13 (IA) in 2011. The second and third batches were exhumed on April 6 (OH) and April 4

Table 2
Origins and burial locations of cultivars and wild biotypes.

Population	Origin[a]	Area (ha)	Coordinates
Cultivar (buried in Columbus, OH, and Ames, IA)			
Kanlow	Northern Oklahoma		
Cave-in-Rock	Southern Illinois		
Sunburst	Southeastern South Dakota		
Nebraska 28	Northern Nebraska		
ADV1	Advanced hybrid cultivar [Summer x Kanlow] (Released as "Liberty" in 2013)		
ADV2	Advanced cultivar selected from Kanlow		
Wild (buried in Columbus, OH)			
OH1	Daughmer Bur Oak Savannah, Crawford Co., OH	0.5	40.7316°, −83.0925°
OH2	South Charleston Railroad Prairie, Clark Co., OH	0.5	39.8183°, −83.6427°
OH3	NASA Plum Brook Research Station, Erie Co., OH	0.4	41.3700°, −82.6477°
IL1	Des Plaines State Conservation Area, Will Co., IL	1.3	41.3891°, −88.1944°
IL2	Goose Lake Prairie State Natural Area, Grundy Co., IL	2.5	41.2155°, −88.1950°
IL3	Midewin National Tallgrass Prairie, Will Co., IL	2.8	41.3472°, −88.0136°
Wild (buried in Ames, IA)			
IA1	Doolittle Prairie State Reserve, Story Co., IA	16	42.1427°, −93.5875°
IA2	Jewell old RR, Hamilton Co. IA	0.2	42.1905°, −93.4616°
IA3	Blairsburg RR & 212th St. Hamilton Co. IA	0.4	42.4822°, −93.6302°
IA4	Honic Slater, Story Co. IA	2.5	41.8872°, −93.6583°

[a] Origins of commercially available cultivars were referenced from www.nativeseednetwork.org.

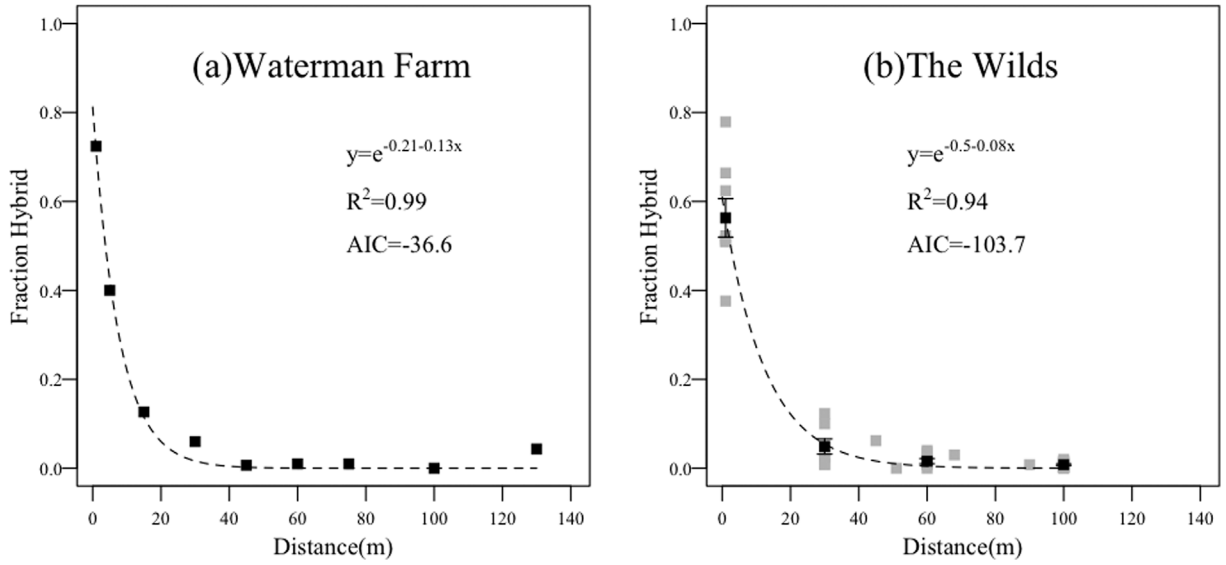

Fig. 3. Fraction hybridization (mean ± SE) observed at each recipient distance at (a) Waterman Farm and (b) The Wilds. The black squares indicated the average fraction hybridization whereas the gray square represented data points at a given recipient distance. The exponential models were the best fit at both locations. At Waterman Farm, data were based on seeds pooled from each recipient distance (n = 9); therefore, no SE was provided. At The Wilds, data were based on multiple recipient triplets at a given recipient distance (see Materials & methods section).

(IA) in 2012, and on April 8 in 2013 at both locations. Once exhumed, seed strips were wrapped with moistened paper towels, sealed in zip bags, and promptly stored in a 4 °C cold room for further examination.

2.2.2. Germination and dormancy tests

After each burial period, recovered seeds were subject to germination and tetrazolium (*2,3,5-triphenyl tetrazolium chloride*, (TTC)) tests for viability and dormancy. Recovered seed strips were rinsed with water, soaked in 5% bleach solution for 5 min, and triple rinsed with water before seeds were removed from packets for examination. Seeds that were hollow, which appeared to have germinated, or appeared mushy were considered nonviable. The remaining seeds were germinated on moistened blotting paper in germination boxes for fourteen days. The germination test took place in a greenhouse with an average temperature of 25 °C with day/night fluctuation in the Biological Science Greenhouse of OSU. We counted and removed germinated seeds during the duration of germination test.

Seeds that failed to germinate during the period of germination test were further tested with TTC for dormancy. We removed part of the endosperm of each seed with a scalpel and stained the embryo ends in 1% TTC solution at room temperature (25 °C) for eight hours in the dark. Seeds with a fully stained embryo were considered dormant, whereas seeds with an unstained embryo were considered nonviable. If embryo and endosperm of a seed were both stained, which is most likely due to fungal infection, we considered the seed nonviable. In the case of a partially stained embryo, we consulted a standard seed examination handbook published by the Association of Official Seed Analyst to determine its viability [45].

2.2.3. Statistical analysis

We calculated percent dormancy based on the "number of dormant seeds" divided by the "number of viable seeds" of each biotype. Because seed dormancy and viability data were not continuous nor normally distributed, we analyzed the data as binary responses — *dormant* (number of seeds that remained dormant after being exhumed) or *viable* (number of seeds germinated during the germination test OR remained dormant) — with generalized linear mixed effect models (lem4 package) and a binomial setting in R [41]. Because the objective was to test for the effects of seed types and biotypes on longevity, we focused on the analyses during each burial period.

To test for the effects of seed type (cultivar *vs.* wild) and biotype (among cultivars and among wild populations) on seed dormancy, we compared percent dormancy of viable seeds between the two seed types. Seed type was the fixed effect whereas biotype and replicate (ten per burial period) were nested under corresponding seed type as the random effects. Further comparisons of seed dormancy among biotypes within each seed type were also carried out — biotype as the fixed effect and replicate as the random effect. To test for the effects of biotypes on seed dormancy among cultivars, we compared percent dormancy with biotype as the fixed effect and burial location as the random effect. Because the wild biotypes buried at the two sites were different, i.e., OH/IL wild biotypes were only buried in OH while IA wild biotypes were only buried in IA, comparison among wild biotypes were carried out within each site.

3. Results

3.1. Pollen-mediated gene flow

3.1.1. Hybridization rate and confirmation of paternity

Based on alleles amplified by the microsatellite DNA marker, seeds that were screened for these markers were all true hybrids of the cultivar donors and wild recipients (Table 1). Therefore, we assumed that all seeds on recipient plants resulted from the pollen flow from the cultivar donors. Based on X-ray images, hybrid seeds were found at all distances, except for 100 m at Waterman Farm (no seeds were found at this distance), and the hybridization rate declined exponentially as a function of distance at both sites (Fig. 3). The maximum average hybridization rate (72%) at Waterman Farm was at 1 m, and the hybridization rate at 130 m, the maximum distance examined, was 4%. At The Wilds, the average maximum hybridization rate (56%) was at 1 m, and the minimum average rate (1%) was found at 100 m, the maximum recipient distance (Table 1).

3.1.2. Best-fit models and minimum isolation distance

We used the average hybridization rate at each recipient distance as inputs to fit models for Waterman Farm. The average hybridization rate at 1 m, 30 m, 60 m, and 100 m were included for the analysis for The Wilds (Table 1). AIC and regression analysis suggested that negative exponential models were the best fit for the pollen-mediated gene flow

Table 3
Estimation of minimum isolation distances (MID) for pollen-mediated gene flow thresholds at 1%, 0.1% and 0.01% in switchgrass at Waterman Farm and The Wilds.

	Best-fit Model	R2	AIC	MID (1%)	MID(0.1%)	MID (0.01%)
Waterman Farm	$y = e^{-0.21-0.13x}$	0.99	−36.6	34m	52m	69m
The Wilds	$y = e^{-0.5-0.08x}$	0.94	−103.7	51m	80m	109m

curves at both sites (Table 3; Fig. 3). Data collected from Waterman Farm yielded an AIC of −36.3, −11.7, and 31.7 for exponential, power, and logarithmic models, and the R^2 were 0.99, 0.81, and 0.42, respectively. At The Wilds, the AIC were −103.7, −7.3, 95.9, and the R^2 were 0.94, 0.16, and 0.68 for exponential, power, and logarithmic models, respectively. Based on the exponential models, the predicted minimum isolation distance for a 0.01% threshold gene flow was 69 m at Waterman Farm and 109 m at The Wilds (Table 3).

3.2. Seed longevity

3.2.1. Seed viability and dormancy

Overall, wild biotypes had a greater number of viable seeds (germinated plus dormant seeds) than the cultivar counterparts during 2011 and 2013 (Table 4). In OH, all of the wild biotypes remained viable over the span of three years, whereas the number of viable cultivar seeds declined drastically by 2012 (an average of 0.5 ± 0.1 seeds) and reached zero in 2013. In IA, cultivar biotypes also lost seed viability more rapidly than the wild counterparts, although viable seeds

Table 5
Results of generalized linear mixed effect model on percent dormancy of viable seeds between seed types (cultivar *vs.* wild). Statistical significance was observed between seed types in 2011 at both sites (*p*-value in bold texts). No dormant seeds were found in 2012 and 2013 at OH, hence, no data for these two years.

	Effects	Estimate	Standard Error	Z value	*p*-value
Ohio					
2011	intercept	−4.52	0.26	−17.08	< 0.001
	type	1.31	0.29	4.57	< **0.001**
Iowa					
2011	intercept	−5.98	0.74	−8.11	< 0.001
	type	1.55	0.68	2.28	**0.02**
2012	Intercept	−8.3	1.97	−4.23	< 0.001
	type	1.19	1.38	0.86	0.39
2013	Intercept	−6.66	2.11	−3.15	0.002
	type	0.48	1.14	0.42	0.67

Table 4
Average (a) number of viable and dormant seeds by biotype and (b) number of viable seeds and percent dormancy of viable seeds by seed type (cultivar *vs.* wild) during 2011 and 2013. Data are presented as mean ± SE. Each biotype included 10 replicates in each year, except for ADV1 (n = 11) and ADV2 (n = 9) in 2011 at IA.

	2011		2012		2013	
(a) Biotype	viable	dormant	viable	dormant	viable	dormant
Ohio						
cultivar						
KL	24.3 ± 1.4	0.40 ± 0.16	1.2 ± 0.4	0 ± 0	0 ± 0	0 ± 0
CIR	27.8 ± 1.4	0.40 ± 0.22	0.8 ± 0.2	0 ± 0	0 ± 0	0 ± 0
SB	27.4 ± 1.6	0.50 ± 0.27	0.4 ± 0.2	0 ± 0	0 ± 0	0 ± 0
NE28	23.7 ± 1.1	0.20 ± 0.13	0.4 ± 0.3	0 ± 0	0 ± 0	0 ± 0
ADV1	31.3 ± 2.2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
ADV2	13.8 ± 1.5	0.60 ± 0.31	0 ± 0	0 ± 0	0 ± 0	0 ± 0
wild						
OH1	39.2 ± 1.8	2.0 ± 0.33	10.8 ± 3.3	0.1 ± 0.10	0.2 ± 0.2	0 ± 0
OH2	44.7 ± 0.7	0.90 ± 0.46	8.7 ± 3.3	0 ± 0	0.7 ± 0.3	0.10 ± 0.10
OH3	32.7 ± 2.1	4.30 ± 0.91	12.2 ± 3.6	0.6 ± 0.4	1.0 ± 0.7	0 ± 0
IL1	43.6 ± 2.0	1.70 ± 0.63	15.9 ± 6.0	0.2 ± 0.13	0.3 ± 0.2	0 ± 0
IL2	38.7 ± 2.3	1.90 ± 0.41	20.1 ± 4.9	0.4 ± 0.22	0.4 ± 0.2	0 ± 0
IL3	32.7 ± 1.9	1.10 ± 0.35	8.1 ± 3.4	0.2 ± 0.13	1.2 ± 0.6	0.10 ± 0.10
Iowa						
cultivar						
KL	20.3 ± 0.9	0.10 ± 0.10	9.8 ± 1.2	0 ± 0	1.0 ± 0.3	0 ± 0
CIR	20.9 ± 1.8	0 ± 0	17.4 ± 0.9	0 ± 0	8.2 ± 1.4	0 ± 0
SB	18.2 ± 1.1	0 ± 0	14.6 ± 1.2	0 ± 0	7.0 ± 1.1	0 ± 0
NE28	12.2 ± 1.2	0.20 ± 0.20	13.8 ± 1.7	1 ± 1	8.7 ± 1.2	0.10 ± 0.10
ADV1	7.5 ± 1.5	0 ± 0	6.7 ± 1.1	0 ± 0	2.8 ± 0.8	0 ± 0
ADV2	1.1 ± 0.3	0 ± 0	0.2 ± 0.2	0 ± 0	0 ± 0	0 ± 0
wild						
IA1	11.9 ± 2.0	0.10 ± 0.10	6.1 ± 1.1	0 ± 0	4.5 ± 0.7	0 ± 0
IA2	33.6 ± 1.3	0.40 ± 0.16	24.3 ± 2.5	0.10 ± 0.10	14.8 ± 1.4	0 ± 0
IA3	8.5 ± 1.4	0.20 ± 0.13	7.8 ± 0.9	0 ± 0	2.9 ± 0.9	0 ± 0
IA4	43.8 ± 1.4	1.0 ± 0.39	41.4 ± 1.1	0.60 ± 0.22	30 ± 2.6	0.10 ± 0.10

(b) Seed Type	viable	% dormant	viable	% dormant	viable	% dormant
Ohio						
cultivar	24.7 ± 0.9	1.68 ± 0.53	0.5 ± 0.1	0 ± 0	0 ± 0	0 ± 0
wild	38.6 ± 1.0	5.54 ± 0.87	12.6 ± 1.7	2.01 ± 0.78	0.6 ± 0.2	0.33 ± 2.34
burial duration	*48 days*		*413 days*		*780 days*	
Iowa						
cultivar	13.5 ± 1.0	0.26 ± 0.19	10.4 ± 0.9	0.93 ± 0.93	4.6 ± 0.6	0.33 ± 0.33
wild	24.5 ± 2.5	1.41 ± 0.40	19.9 ± 2.4	0.44 ± 0.18	13.1 ± 1.9	0.06 ± 0.06
burial duration	*74 days*		*401 days*		*770 days*	

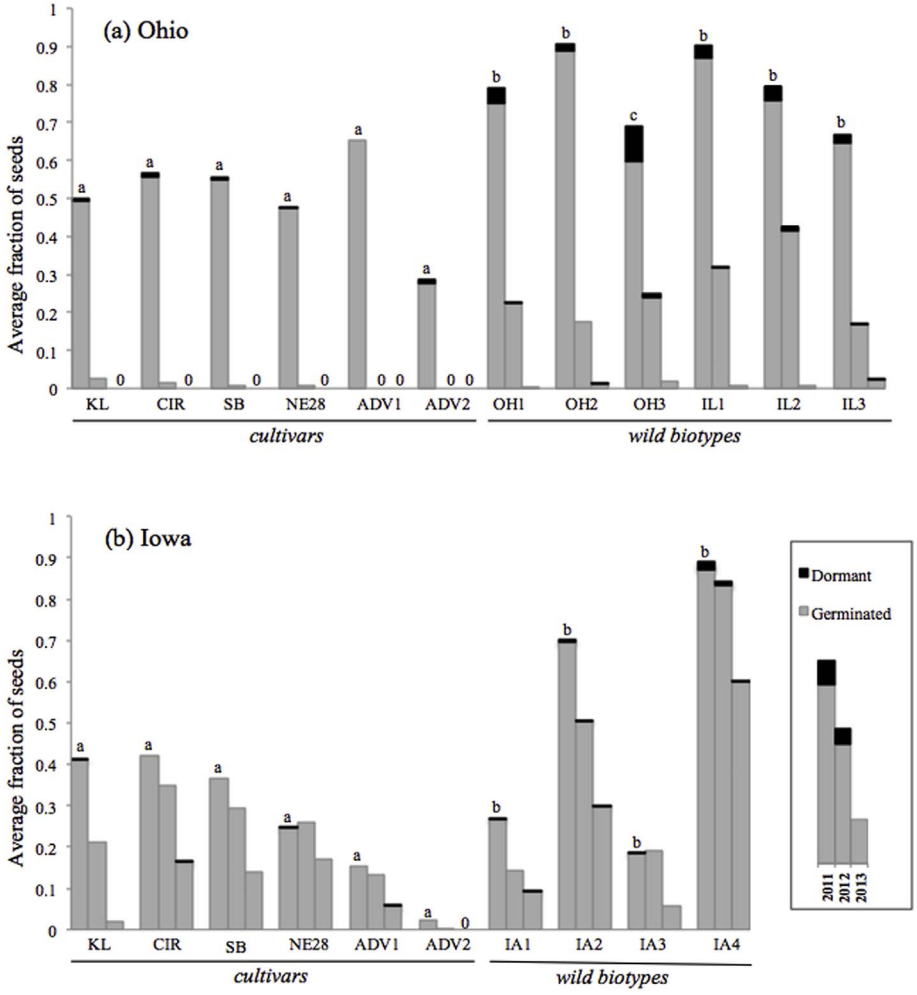

Fig. 4. Average fractions of dormant (black column section) and germinated (gray column section) seeds in (a) OH and in (b) IA during 2011and 2013. The clustered columns represent data of a given biotype in 2011, 2012, and 2013. Data were calculated based on 10 replicates (50 seeds per replicate), with the exception of ADV1 and ADV2 (n = 11 and 9, respectively) in 2011. Zeros indicate that no viable seed was found in the particular year. Wild biotypes had a significantly higher percent dormancy than cultivars in 2011 at both locations ($p < .001$ in OH and $p = .02$ in IA). In 2011, OH3 had significantly higher percent dormancy than any other wild populations in OH ($p < .01$), whereas cultivars were similar in percent dormancy. No significant difference in percent dormancy was detected among wild biotypes or among cultivars in IA during the three years.

were still found in all biotypes by 2013, with the exception of ADV2 (on average, 4.6 ± 0.6 cultivar seeds *vs.* 13.1 ± 1.9 wild seeds).

Regarding seed dormancy, on average, wild biotypes retained a greater number of dormant seeds than the cultivar counterparts (Table 4a). By 2011, wild biotypes had a significantly higher percent dormancy than cultivar counterparts at both locations ($p < .001$ and $p = .02$ in OH and IA, respectively; Table 5; Fig. 4). Among the wild biotypes buried in OH, OH3 had a significantly higher percent dormancy than the rest of the cohort in that year (Fig. 4a). By 2012, all of the cultivar biotypes had completely lost seed dormancy in OH (hence, no statistical analysis were carried out for 2012 and 2013), while the difference in percent dormancy was insignificant between cultivar and wild seeds in IA in the subsequent years ($p = .39$ in 2012 and $p = .67$ in 2013; Table 5; Fig. 4b). There was also no significant difference in percent dormancy among cultivars or among wild biotypes buried in IA from 2011 to 2013.

3.2.2. Effects of burial location

Cultivar biotypes buried in OH had a greater number of viable seeds than in those buried in IA in 2011 (Table 4). On average, 24.7 ± 0.9 cultivar seeds were viable in OH whereas only 13.5 ± 1 cultivar seeds were viable in IA (Table 4b). However, an opposite trend was observed starting in the following year — only four of the six cultivars still retained a small number of viable seeds in OH (an average of 0.5 ± 0.1 seeds), while all cultivars had a higher number of viable seeds in IA (an average of 10.4 ± 0.9 seeds) in that year. By 2013, all cultivar biotypes had lost seed viability in OH, whereas only one cultivar biotype reached zero viability in IA (an average of 4.6 ± 0.6 seeds). Concerning dormancy, a small number of cultivar seeds remained dormant at the end of the first burial period at both locations. Burial location had a significant impact on percent dormancy of the cultivar seeds in 2011 ($p = .04$). However, all cultivar biotypes lost dormancy in the subsequent year, except for NE28 in IA, hence, no statistical analysis was carried out for 2012 and 2013.

4. Discussion

4.1. Pollen-mediated gene flow

4.1.1. Pattern and extent

The distribution of pollen-mediated gene flow in switchgrass was leptokurtic, and the pattern was best described by the exponential model at both sites. The pattern was similar to reported results of other wind-pollinated species such as oilseed rape, *Brassica napus* [46], barley, *Hordeum vulgare* [47], and sorghum, *Sorghum bicolor* [48]. The furthest distance tested was 100 m and 130 m at The Wilds and Waterman Farm, respectively. However, gene flow could still occur at a greater distance, although the frequency would continue to decline.

Variation of gene flow pattern was observed between sites. Waterman Farm had a higher hybridization rate at 1 m compared to The Wilds, but the fraction declined more drastically at Waterman Farm than that in The Wilds. The variation between sites could be attributed to several factors. First, a greater number of donor panicles (4903 *vs.* 2289) was present at Waterman Farm than at The Wilds (Appendix A). The greater size of pollen source may have resulted in a higher hybridization rate at 1 m away from the donors. Second, although there

was a greater ratio of donor-to-recipient panicles at Waterman Farm than at The Wilds (3.5:1 *vs.* 1.4:1), more pollen travelled further at the latter location. One explanation may be that the plot at The Wilds sat on a hill with a more open landscape, whereas the plot at Waterman Farm was planted amongst soybeans and to the south of a tree line. The existence of the mixture of vegetation at Waterman Farm may have disturbed wind currents.

The experimental design may also have an impact on the estimation of hybridization rate. First, our plot at The Wilds was a circular design sitting on an open landscape, and the estimation of hybridization rate was based on data across cardinal directions. On the contrary, the plot at Waterman Farm was a rectangular design with all recipient plants lining to the east of the donor plants; therefore, data at this location only presented the scenario to the east side of the donor plants. In addition, because hybridization rate was calculated based on recipient panicles that flowered during the peak overlap period of the donor and recipient ramets, the actual hybridization rate is expected to be lower throughout the season if all panicles were sampled. Second, we used clonal, self-incompatible recipient plants, which did not allow for effective cross-pollination among recipients. If recipients had been able to cross-pollinate, fertilization from more distant pollen donors is expected to occur at a lower frequency [49]. Therefore, our estimation of pollen-mediated gene flow from the cultivar donors to wild recipients was probably appropriate for very isolated wild plants, but higher than expected in wild populations with a large number of neighboring individuals. Thus, the isolation distance that we reported for specific thresholds are likely to be conservative.

Our findings suggest that crop-to-wild hybridization in switchgrass could occur beyond 100 m from a relatively small donor source (~100 plants). Our findings of pollen-mediated gene flow in switchgrass are consistent with those of Millwood et al. [28], who employed a similar design to study crop-to-crop gene flow in switchgrass. Based on our estimation, to prevent pollen-mediated gene flow to conspecific relatives, at least 109 m of minimum isolation distance is recommended for a small field trial of transgenic switchgrass in an open field. In commercial scale planting, a much larger pollen source and a higher frequency of pollen-mediated gene flow are expected. Therefore, a greater minimum isolation distance is desirable if the goal is to minimize gene flow [50–54] (but see Ref. [55]). As discussed earlier, abiotic conditions should also be taken into account — when transgenic crops are grown under conditions more optimal for pollen dispersal, a greater minimum isolation distance is also recommended.

4.1.2. Implications for transgenic switchgrass field trials

The current minimum isolation distance for “non-transgenic” seed purity standards may not be sufficient for transgenic switchgrass. According to the USDA, a minimum isolation distance of 274 m is required to produce foundation seeds of cross-pollinating grass species [56]. Data presented in this study were based on panicles collected at the end of the first growing season when plants were small in size. In the subsequent year, each donor ramet, on average, produced 1.5 to 2 times more panicles than the first year — 60.3 ± 3.1 *vs.* 29.9 ± 2.8 panicles at Waterman Farm; 32.5 ± 2.4 *vs.* 21.6 ± 1.6 panicles at The Wilds (second year data not shown). Therefore, frequency of pollen-mediated gene flow was expected to be higher and to reach a greater distance due to a larger amount of pollen produced in the second year. A much greater minimum isolation distance beyond 109 m, or even 274 m, may be required to minimize gene flow from transgenic switchgrass to neighboring non-transgenic counterparts.

4.2. Seed longevity

4.2.1. Longevity of wild vs. cultivated seeds

Seed dormancy allows a species to persist in the seed bank during conditions unfavorable for germination, and a lower level of seed dormancy is usually observed in domesticated species compared to their wild counterparts as a result of directional selection or pleiotropic effects [57–60]. Switchgrass has been traditionally bred for feedstock, wildlife habitats, and soil erosion control, and minimal site preparation and labor is involved. Consequently, quick establishment is crucial for the success of a switchgrass cultivar. It is possible that selection for traits associated with rapid establishment may have lead to the loss of seed dormancy during the process (see review in Ref. [61]). Our study demonstrated that switchgrass cultivars lost dormancy within a short period of burial. Therefore, if new bioenergy varieties are bred based on existing cultivars, they are likely to possess seed longevity and dormancy similar to the current cultivars, assuming there is no major change in genes responsible for these characteristics. Furthermore, if pollen-mediated gene flow occurs between new bioenergy cultivars and wild relatives, seed dormancy and longevity of the resulting crop-wild hybrid offspring are likely to be intermediate between the cultivar and wild parents, although the direction of gene flow may influence the characteristics of crop-wild progenies. For instance, crop-wild hybrid canola seeds with wild maternal background were reported to have higher dormancy than those with cultivar maternal background [62]. Fitness comparison of such crop-wild hybrid and their parents should be further investigated to complement current ecological understanding of switchgrass.

Besides domestication, the origin of seeds could play a key role in dormancy and persistence in the soil. Studies have suggested that the minimum temperature requirement for switchgrass germination varies, and it is highly dependent on the latitude of the origin — southern genotypes generally require a higher minimum temperature than those from northern latitudes [63–65]. In this study, all of the cultivars originated from similar latitudes (Illinois, Nebraska, and Oklahoma) with the exception of SB (from S. Dakota), although SB exhibited similar seed viability compared to other cultivars in this study. Nevertheless, new bioenergy cultivars are likely to originate from a broader range of geographical regions; hence, future assessments of seed longevity should still take origin and planting locations into account.

4.2.2. Effects of burial location

Abiotic conditions such as soil temperature and soil moisture have been linked to variation of seed longevity in the soil — a seed is generally more likely to persist in the soil for a longer period in a drier, cooler environment than in a damper, warmer environment [66]. Contrary to the common trend, cultivars buried in OH (a damper, warmer climate) retained significantly higher percent dormancy than those buried in IA (a drier, cooler climate) by the end of the first burial period in our study. The higher percent seed dormancy in OH may be explained by the shorter burial period than that in IA (48 days *vs.* 74 days). In addition, the average soil temperature in OH was lower than in IA during this period (5.5 °C in OH *vs.* 6.3 °C in IA), and seeds buried in OH experienced less frost-free days than those buried in IA (4 days *vs.* 19 days; Appendix B). However, in the subsequent year, all cultivars lost dormancy, except for NE28 in IA, which had dormant seeds throughout the experiment. A similar trend was observed in seed viability. In 2011, a greater number of cultivar seeds were viable in OH than in IA, but fewer cultivar seeds were viable in OH in comparison to IA by 2012.

In addition to climatic conditions, the fate of switchgrass seeds depends on the location and environment in which they land. In this study, we buried seeds at the depth of 20 cm to mimic the common depth of tilling, which only reflected one of the “dispersal” scenarios. It was unlikely that these seeds would emerge naturally given the burial depth. However, certain biotypes, especially wild biotypes, may persist in the soil for at least two years, as suggested in our study. If they are brought close to the soil surface, it is possible that they could still germinate, assuming they have remained dormant prior to the disturbance.

5. Conclusions

Based on our study, a small planting of ~100 switchgrass plants would require approximately 109 m isolation distance to meet the 0.01% gene flow threshold, but this distance is likely to at least double as plants mature. Current switchgrass cultivars are not likely to persist in the soil seed bank for more than three years, and only a small number of wild seeds could remain viable in a cooler and drier environment beyond this time frame. Therefore, screening of volunteer seeds in field trials involving transgenes should be carried out for at least three years after the removal of transgenic plant materials. In the case of commercial-scale planting with a much larger pollen and seed reservoir, additional preventive strategies, i.e., inducing male sterility, selecting for late flowering genotype, and prohibiting flowering, would be required to effectively prevent or mitigate gene flow from transgenic switchgrass to non-transgenic and wild relatives.

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Appendix A. Number of panicles (mean ± SE) produced by pollen donors and recipients at Waterman Farm and The Wilds. C:W indicates the ratio of total panicles produced by cultivar and wild clones

	Waterman Farm			The Wilds		
	Donor (cultivar)	Recipient (wild)	C:W Ratio	Donor (cultivar)	Recipient (wild)	C:W
(a) No. of panicles/plant	29.9 ± 2.8 (n = 20)	9.3 ± 1.7 (n = 40)		21.6 ± 1.6 (n = 20)	14.5 ± 1.5 (n = 117)	
(b) No. of plants	164	153		106	117	
Total panicles produced (a x b)	4903	1422	3.5:1	2289	1696	1.4:1

Appendix B. Records of soil temperature and precipitation during 2011 and 2013 at the burial locations in OH and IA. Data were extracted from the Ohio Agricultural Research and Development Center weather system (http://www.oardc.ohio-state.edu/newweather/) for the OH site (Columbus station) and from the Iowa State University AgClimate Network (http://mesonet.agron.iastate.edu/agclimate/) for the IA site (Ames station)

Abiotic Conditions	Columbus, OH	Ames, IA
Soil Temp. (°C, mean ± SE)		
1st burial period	5.5 ± 0.6	6.3 ± 0.6
no. of frost-free days	4 days	19 days
2nd burial period		
warmest months (Jun–Aug)	25.7 ± 0.2	23.9 ± 0.3
coldest months (Dec–Feb)	4 ± 0.2	−0.2 ± 0.1
no. of frost-free day	203 days	195 days
3rd burial period		
warmest months (Jun–Aug)	25.2 ± 0.2	26.7 ± 0.4
coldest months (Dec–Feb)	4 ± 0.2	−1.2 ± 0.3
no. of frost-free days	187 days	176 days
Cumulative Precipitation (cm)		
1st burial period	23.1	13.4
2nd burial period	115.5	62.8
3rd burial period	74.2	50.4

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