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Switchgrass (Panicum virgatum L.) Distribution, Genetic Diversity, and Pollen-Mediated Gene Flow

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Switchgrass (*Panicum virgatum* L.) is a North American grass being optimized to serve as a biofuel crop. The cultivation of switchgrass carrying novel genes and traits has made ecological risk assessment (ERA) research critical for predicting the effects of pollen dispersal and pollen-mediated gene flow on local switchgrass populations and plant communities. The goals of this research were to: 1) Understand *Panicum* species distribution, habitat types, and plant communities; 2) Create a Lagrangian model of pollen dispersal informed by data on pollen longevity, size, and wind fields; 3) Determine the potential for interspecific hybridization between switchgrass and other *Panicum* species; 4) Characterize the genotypes in switchgrass populations in coastal grasslands and inland road verges. All studies were conducted in the northeastern U.S. (Ecoregion Level III: Northeastern Coastal Zone, Northeastern Highlands). In the first study, a botanical survey showed that switchgrass populations were common in three coastal habitat types (semi-natural, human impacted, and roadside) as well as two inland habitats (roadside and wastelands) outside of their expected range. In the second study, pollen longevity, pollen size, and wind fields were used to parameterize a Lagrangian mathematical model to predict pollen dispersal. Switchgrass pollen grains entrained in light, buoyant winds were predicted to travel up to approximately 3.5 km. A model
incorporating stronger, directional winds predicted pollen dispersal up to about 6.5 km. In the third project, switchgrass flowers were crossed with flowers from four other *Panicum* species but did not produce hybrid progeny. However, technical challenges prevented robust analysis of interspecific sexual compatibility. In the last study, switchgrass plants collected from inland roadsides and coastal habits were analyzed using their DNA and simple-sequence repeat (SSR) molecular markers. The switchgrass plants in the study were genetically diverse; the majority of inland individuals were identified as Upland octoploids genotypes, while the majority of coastal individuals were identified as Lowland tetraploid genotypes. Collectively, these projects suggest that pollen-mediated gene flow from switchgrass biofuels plantations could potentially alter the genetics of local coastal populations, as well as the composition of natural or roadside plant communitie
Switchgrass (*Panicum virgatum* L.) Distribution, Genetic Diversity, and Pollen-Mediated Gene Flow

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A Dissertation
Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Connecticut 2014
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Doctor of Philosophy Dissertation

Switchgrass (*Panicum virgatum* L.) Distribution, Genetic Diversity, and Pollen-Mediated Gene Flow

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Chapter 1

Introduction

Humans have been manipulating the genetics of plants through domestication and breeding for over 10,000 years, but it is only in the last few decades that this modification has taken the form of direct gene transfer between organisms without sexual reproduction (Gepts, 2002). These new technologies have been called genetic engineering (GE) and have created concerns about possible unintended consequences for human health and the environment (Craig, Tepfer, Degrassi, & Ripandelli, 2008). These concerns have prompted regulatory bodies in the US and other countries to pursue ecological risk assessment (ERA) to predict potential risks from a GE crop (Conner, Glare, & Nap, 2003). ERA is a complex undertaking which must, in order to be robust, take into account crop biology, the new genes and traits, and the environment where the GE crop will be grown. The overarching goal of this research was to support the ERA of Panicum virgatum L. (switchgrass) with novel traits in the northeastern US.

Panicum virgatum is a warm season, perennial, bunchgrass native across eastern and central North America (Figure 1). The species is strongly self-incompatible and wind-pollinated (J M Martinez-Reyna & Vogel, 2002). In the northeastern US, its distribution before European colonization is thought to have been the narrow inland zone adjacent to the coastal salt marsh, although much of this habitat has now been altered by human settlement (Niering & Warren, 1980). Besides being part of native coastal and prairie plant communities, switchgrass has been used as a forage crop for livestock (Mclaughlin & Kszos, 2005). In recent years it has grown in popularity as a species for
conservation plantings, erosion control, and ornamental beauty as well as a model biofuel feedstock (E. Heaton, 2004; Parrish & Fike, 2005; Wright & Turhollow, 2010).

When the U.S. Department of Energy’s (DOE) Herbaceous Energy Crops Program began its research in the mid-1980s, switchgrass was selected as a potential crop for biofuels production due to its high biomass yields across a variety of environmental conditions (Parrish & Fike, 2005). It was chosen as a model bioenergy species not only for its high biomass yields, but also because it had low fertilization requirements. Furthermore, switchgrass was found to have positive environmental effects such as preventing soil erosion and improving soil quality while providing cover for wildlife (McLaughlin & Walsh, 1998). These effects were due in large part to switchgrass’s perennial lifecycle and extensive root mass. As a perennial, biomass production could proceed for many years before soil disturbance and reseeding were necessary (Carroll & Somerville, 2005). In addition, switchgrass’s cold hardiness and drought tolerance could allow its cultivation on marginal land not currently used for crop production (Wright & Turhollow, 2010). This might be a particularly important due to recent concerns over the potential for biofuel feedstocks to compete with traditional food crops (e.g. corn) for arable land driving up food prices (Tilman et al., 2009).

After being identified as an ideal feedstock for lignocellulosic ethanol production, switchgrass became a target for transgenic manipulation (Wright & Turhollow, 2010). The first experimental field test permits for GE switchgrass were issued in 2008 for varieties with enhanced drought tolerance and male sterility. Since there first field tests, transgenic switchgrass varieties with a number of altered traits have been tested. New traits have affected crop performance (e.g. alterations to senescence, stalk strength,
drought tolerance, nitrogen use efficiency, and growth rate) and biofuel production (e.g. increased yield, biomass conversion efficiency, and reduced lignin) (Simmons, Loqué, & Ralph, 2010). As of this writing, a total of 24 experimental field trial permits have been approved in the US (Information Systems for Biotechnology, 2014). In addition, at least one transgenic line of switchgrass produced by Ceres, Inc “TRG 1.0 1B” has been deemed by USDA not a plant pest and therefore not under their regulatory authority (Ceres, Inc, 2010). Thus, there is a strong rationale in favor of ERA for switchgrass with novel genes and traits.

Ecological risk can be defined as the product of a hazard (an adverse environmental impact) and an exposure (a mechanism or route by which the hazard is experienced) (Auer, 2008; Hayes, 2004). Some of the potential hazards that have been discussed for GE switchgrass include: 1) the loss of genetic diversity, unique alleles, and adaptability in native switchgrass populations; 2) creation of new weedy or invasive genotypes; or 3) direct or indirect negative effects on communities or non-target organisms (Barney and DiTomaso, 2008; DiTomaso et al., 2007; Raghu et al., 2006; Wolt, 2009). Although the characterization of potential environmental hazards is very important, the focus of this research has been on understanding the potential exposure pathways involving switchgrass distribution and gene flow.

Taxonomy and Distribution

_Panicum_ is a member of the family Poaceae. At the subfamily level, _Panicum_ falls within the Panicoideae, a very large subfamily that includes a number of the world's economically important crops (e.g. _Panicum miliaceum_, _Zea mays_, and _Saccharum officinarum_). _Panicum_ taxa can be found on all continents except Antarctica (Giussani
et al., 2001). *Panicum* is one of the largest genera in the Poaceae with more than 400 species (Webster, 1988). Although most *Panicum* species are diploid or tetraploid, many species contain multiple ploidy levels (Martinez-Reyna & Vogel, 2002; Brown, 1948).

The taxonomy of *Panicum* has been controversial and under revision for more than 100 years (Hitchcock & Chase, 1910). A number of recent molecular phylogeny studies have suggested that the genus remains polyphyletic even after numerous realignments (Aliscioni et al., 2003; Duvall et al., 2001; Giussani et al., 2001; Zuloaga et al., 2000). In contrast, a study conducted by Giussani et al. (2001) reaffirmed the monophyletic nature of the subgenus *Panicum*. In addition, the subgenus *Dichanthelium* was elevated to the generic level.

Many *Panicum* species are confined to tropical regions (Barkworth, Anderton, Capels, Long, & Piep, 2007), so it is not surprising that the temperate northeastern United States (i.e. the area represented by Maine, Vermont, New Hampshire, Massachusetts, Connecticut, and Rhode Island) is home to just nine species of *Panicum* (Haines, 2011). The northeastern *Panicum* species include: *P. virgatum, P. amarum, P. rigidulum, P. verrucosum, P. capillare, P. dichotomiflorum,* and *P. miliaceum*. Three of the native *Panicum* are generally found in natural areas (*P. amarum, P. rigidulum, P. verrucosum*); two native species are often seen as weedy populations (*P. capillare* and *P. dichotomiflorum*); and one non-native species can be found as either a crop or weed (*P. miliaceum*, Proso millet). *P. virgatum* can fall into all three of these categories depending on the environmental context. Of all the *Panicum* species in the Northeastern United States, *P. amarum* (Bitter Beachgrass) is by far the most similar to switchgrass with regional flora suggesting that the two species intergrade (Barkworth et al. 2007; Haines
The close relationship between *P. virgatum* and *P. amarum* was first studied in detail by Palmer (1972).

**Genetics of Switchgrass**

Switchgrass is commonly divided into two broad ecotypes, Upland and Lowland. Lowland ecotypes are distinguished by their larger stature and are typically tetraploids, while Upland ecotypes are somewhat smaller and can be either tetraploid or octaploid (J M Martinez-Reyna & Vogel, 2002; Porter, 1966).

Studies of population structure and genetic diversity in switchgrass have been conducted with number of molecular techniques stretching back to the 1940’s when root tip squashes were used to determine chromosome counts. Early reports suggested wide variation in chromosome number (W. V Brown, 1948; Nielsen, 1944). Later, Porter (1966) described the familiar modern understanding of Lowland tetraploid (2n = 4X = 36) plants and Upland tetraploids and octoploids (2n = 8X = 72). Riley & Vogel (1982) produced chromosome counts of the named cultivars though it should be noted that several of these do not agree with the modern literature. Wild plants can vary in ploidy from diploid (2n=18) to duodecaploid (2n=108), although tetraploid and octoploid lines are most common (Rogers & Mantalvo, 2004). Switchgrass is now understood to be an allopolyploid species with a base chromosome number of nine (Costich et al. 2010). Tetraploids display disomic inheritance with three genotype classes (AA, AB, BB), and octoploids have four homologous chromosomes and five genotype classes (AAAA, AAAB, AABB, ABBB, BBBB)(Grabowski, Morris, Casler, & Borevitz, 2014; Okada et al., 2010).
Modern interest in switchgrass biotechnology has encouraged a number of molecular studies into the genetic diversity and genetic resources of the species. The methodology that has been used includes: random amplified polymorphic DNA (RAPD; Gunter et al., 1996; Casler et al., 2007); restriction fragment length polymorphism (RFLP; Hultquist et al., 1996; Missaoui et al., 2006); amplified fragment length polymorphism (AFLP; Todd et al., 2011); simple sequence repeats (SSRs; Cortese et al., 2010; Narasimhamoorthy et al., 2008; Zalapa et al., 2011). In addition, new genomic approaches using high-throughput sequencing have also been used to elucidate population structure and genetic diversity. These studies have reaffirmed the ecotype groups found by older methods as well as lending further evidence to the idea that the northerly migration of the species from glacial refugia resulted in the modern ecotypes (Lu et al., 2013; Morris et al., 2011). The Lowland and Upland ecotypes are thought to correspond broadly with the two glacial refugia posited by McMillan (1959). Since the last ice age, switchgrass has radiated out into five main ancestral lineages. The Upland ecotype can be divided into a northern tetraploid lineage stretching from North Dakota to New York and two octoploid lineages developing from the Great Plains or Gulf Coast. The Lowland ecotype separated into two lineages, one from the southern Great Plains and the other from the eastern Gulf Coast (Zhang et al. 2011a). Zhang et al. (2011a) suggested that the eastern Gulf Coast is the primary center of diversity in switchgrass though it is possible that this concept is an artifact of insufficient sampling of the western Gulf coast. The identification of a phenotypically Lowland plant with an octoploid genome in the eastern Gulf Coast lends credence to the idea of high genetic diversity in the east.
There is no information about the genetics of native switchgrass north of New York and Long Island. This is because previous studies have not included samples from the northeastern US and Canada (Cortese et al., 2010; Lu et al., 2013; Zhang et al., 2011a). Cortese et al. (2010) collected switchgrass in New York and these plants grouped most closely with ‘High Tide’ (L4X-A in this study), though that relationship did not hold when morphological markers were also included in their analysis. Lu et al. (2013) collected switchgrass from New York including Long Island and suggested that these individuals belonged to a “Lowland 4x Northeast” group. However, this study could not make a connection with the L4x ‘Florida Clade’ identified by Zhang et al (2011a) because it did not include accessions from the Southeastern Atlantic or Gulf Coasts (Florida, South Carolina, North Carolina). The study by Zhang et al. (2011a) included samples for the Southeastern region; however they did not include accessions north of New York City and New Jersey. Therefore, the lack of accessions from our region provided a strong rationale for botanical surveys and genetic studies.

Gene Flow

Vertical gene flow is defined as the movement of genes from one population to another followed by introgression into the receiving population (Andow & Zwahlen 2006; Stewart et al. 2003). Gene flow is widely regarded as an important aspect of ERA for GE crops and has been studied extensively (Dale et al. 2002; Ellstrand 2003; Auer 2008; Snow 2002). Gene flow requires that plants be genetically and reproductively compatible, have synchronous flowering, and the production of viable seed. Gene flow events can be followed by introgression, the fixation of new genes in receiving populations (Ellstrand, 2003). Another important factor in gene flow is fitness, the ability
of an individual to pass its genes to subsequent generations through viable offspring (Snow et al. 2003; Ellstrand 2003; Tepfer 2002).

Gene flow in switchgrass could occur through the movement of pollen, seed or vegetative propagules (rhizomes). Very little is known about switchgrass seed dispersal although long distance seed dispersal is most likely via human transport or water dispersal as switchgrass seed lacks specialized dispersal mechanisms (Kwit & Stewart 2012). To my knowledge, there has been no research on gene flow in switchgrass via rhizomes. However, various studies have focused on gene flow on the evolutionary time scale in switchgrass (see section above on genetics) and this would include all forms of gene flow over time. The dispersal of pollen in anemophilous species has been elucidated through mathematical models of particle movement using wind field and pollen capture data (Wang & Yang 2009; Aylor & Boehm 2006; Arritt et al. 2007). Among the factors affecting successful pollen-mediated gene flow are pollen size, density, viability, and longevity of the pollen. The duration of pollen viability has been estimated by testing the ability of pollen grains to produce seeds, in vitro germination, or the application of vital stains (Dafni and Firmage, 2000). Various studies have incorporated the effect of stress (e.g UV radiation, humidity, temperature) on pollen viability (Ge et al., 2011; Bragg, 1969; Aylor, 2003; Fonseca and Westgate, 2005). Switchgrass may be particularly prone to gene flow and transgene escape because: it has undergone relatively little domestication (Zalapa et al. 2011); is closely related to three aggressive weeds (Colosi & Schaal, 1997; Uva, Neal, & Ditomaso, 1997); is outcrossing and self incompatible (Martinez-Reyna & Vogel, 2002); shares habitat with wild relatives (Barkworth et al., 2007). This research has addressed some of the critical aspects of pollen-mediated gene
flow in switchgrass including pollen biology, dispersal, and distribution of receiving populations.

Dissertation Chapters

The research projects undertaken for this dissertation were designed to support ecological risk assessment for switchgrass carrying novel genes and traits. This information is critical for decision making by stakeholders including government regulators, plant breeders, land managers, seed producers, biotechnology companies, and farmers. The following chapters present four major research areas:

Chapter 2) The first study was a botanical survey designed to address the following ecological questions in natural, cultural and agricultural landscapes: (1) What is the current distribution of Panicum species with respect to inland and coastal ecoregions, habitat types, and plant community assemblages in the northeastern U.S.?, (2) Do Panicum species co-occur in plant communities such that interspecific pollen-mediated gene flow could be promoted?, and (3) Are management or disturbance events commonly associated with Panicum populations?

Chapter 3) The objective of the second study was to characterize switchgrass pollen traits and apply that information in modeling pollen dispersion using a Lagrangian approach. Sonic anemometers measured wind fields at two discrete locations and times, creating two contrasting scenarios for modeling (light wind with buoyant turbulence or stronger, pressure-driven, non-turbulent winds).

Chapter 4) The third study was designed to understand interspecific compatibility between switchgrass and four other Panicum species. The major question was can
switchgrass produce viable hybrid seed when crossed with con-generic species from North Eastern North America?

Chapter 5) The purpose of the forth study was to analyze the DNA from switchgrass plants collected in inland and coastal ecoregions. PCR for molecular markers produced DNA fragments that were used to identify genotypes and similarities with well known Upland and Lowland ecotypes. The goals were to: 1) determine if switchgrass plants observed in the Long Island Sound Coastal Lowland ecoregion were native genotypes from pre-settlement switchgrass populations, and 2) determine if plants observed in road verges were the result of range expansion from coastal populations or cultivars developed from non-local gene pools.


Mutegi, E., Stottlemyer, A. L., Snow, A. a., & Sweeney, P. M. (2013). Genetic Structure of Remnant Populations and Cultivars of Switchgrass (Panicum virgatum) in the


Figure 1: North American native range of *Panicum virgatum* (USDA).
The intersection of ecological risk assessment and plant communities: an analysis of *Agrostis* and *Panicum* species in the northeastern U.S.

(Presented in the format as published in Plant Ecology)

Abstract:

Ecological risk assessments for grass species with novel traits are advisable, or even required, in order to identify potential environmental harms prior to large-scale cultivation. Credible risk assessments are built upon knowledge of the communities that could be negatively affected by crop-to-wild gene flow, new weeds, or invasives. This study focused on two cultivated grasses with different life histories: the exotic, weedy *Agrostis stolonifera* (creeping bentgrass) and the native *Panicum virgatum* (switchgrass). Vascular plant communities were analyzed in 190 transects (50 m) in ten habitat types across two ecoregions (inland and coastal) in the northeastern U.S. Ordination plots and dendrogram analysis showed clustering of inland plant community assemblages within habitat types, while coastal plant communities were similar across the habitats studied. *Agrostis* and *Panicum* species had unequal distribution across the habitat types and ecoregions, with *Agrostis* species more common in the inland ecoregion and habitats receiving moderate management or disturbance events. In both ecoregions, *A. stolonifera* had high co-occurrence values with other exotic *Agrostis* species, suggesting potential for interspecific gene flow. Inland, *P. virgatum* was observed in roadside and wasteland habitats, but was distributed equally in the three coastal habitats studied. Co-occurrence between *P. virgatum* and congenerics was infrequent, although one transect had both *P. virgatum* and the state-listed coastal species *P. amarum*. Plant community and distribution data of this type provide the basis for ecological risk assessments, predictions about pollen-mediated gene flow, containment strategies, and geographic exclusion zones.
Keywords: habitat, plant community, Agrostis, creeping bentgrass, Panicum, switchgrass, ecological risk assessment

Abbreviations: genetically-engineered, GE; Lower New England Ecoregion subecoregion Southern New England Coastal Hills and Plains, CHP; North Atlantic Coast Ecoregion, subecoregion Southern New England Coastal Lowland, CL; ecological risk assessment, ERA.

Introduction

Grasses provide much of the world’s food, but they also act as weeds and invasive species in diverse agricultural, natural, and cultural landscapes. In the U.S., a national energy initiative has identified a number of C₄ grasses as potential biofuel feedstocks, but some of these species are considered weedy or invasive plants (Raghu et al. 2006). The large-scale production of these plants could lead to management or biological problems. The development of genetically-engineered (GE) grasses have raised concerns about the downstream harm from crop-to-wild or crop-to-weed gene flow, especially if the novel phenotype increases stress resistance, fecundity, or overall fitness (Andow and Zwahlen 2006; Stewart et al. 2003; Wilkinson and Tepfer 2009). In addition, preventing gene flow in wind-pollinated grasses poses a distinct challenge, and the containment problem increases when sexually-compatible species (receiving populations) exist in natural, agricultural, or cultural landscapes (defined as properties representing the combined work of nature and man, UNESCO, 2010). The escape of glyphosate-resistant Agrostis
*stolonifera* (creeping bentgrass) in the western United States provides an example of the challenges involved in biocontainment (Reichman et al. 2006; Watrud et al. 2004; Zapiola et al. 2008).

The harmonization of policy for GE crops across many countries has resulted in a regulatory process that requires the construction of predictive ecological risk assessments (ERA) prior to commercialization (Andow and Zwahlen 2008; Auer 2008; Craig et al. 2008; Nap et al. 2003). Risk analysts and regulators generally assess GE crops using a case-by-case approach with detailed knowledge about the biology of the crop, the biotechnology-derived trait, and the environment in which the crop will be grown (EPA 1998). The biggest challenges today in ERA are the identification of potential harms to valued communities and ecosystems (problem formulation), and the conduct of research that allows quantification of future ecological changes (Wilkinson and Tepfer 2009). This is not a theoretical concern; non-native grass invasion has contributed to the decreased of abundance in birds and arthropods compared to native grassland communities (Flanders et al. 2006).

In the case of ERA for cultivated grasses, understanding plant community dynamics is important to characterize direct or indirect harms to non-target species and the distribution of receiving populations for crop-to-wild gene flow. Unfortunately, regional floras and botanical references are generally insufficient to assess these risks because they are often incomplete, out-of-date, or they do not include information about cultural or agricultural landscapes (Barkworth et al. 2007; Magee and Ahles 2007; Gleason and
In addition, botanical references typically have no information about the co-occurrence of closely-related species that could favor interspecific gene flow. This information is critical for the design of exclusion zones, regions where specific GE crops are not permitted because of crop-to-wild gene flow (McGinnis et al. 2010). Our project was designed to provide information about the distribution, habitat types, and plant communities for two grass species that have been modified through traditional breeding and biotechnology to create novel traits for the U.S. market: *Agrostis stolonifera* (creeping bentgrass) and *Panicum virgatum* (switchgrass).

*Agrostis stolonifera* is a cool-season, perennial grass planted in golf courses and other cultural landscapes in the U.S. (Beard 2002). In general, *A. stolonifera* in the U.S. is considered an introduced species, but some sources cite specific ecotypes (or populations) as native (Barkworth et al. 2007). Field test permits were issued for GE *A. stolonifera* (2002-2008) tolerant to herbicides, insects, drought, salt, heat, disease, and shade (ISB 2010). Male sterility and altered plant morphology have also been tested in experimental field trials. Herbicide-resistant *A. stolonifera* has generated the most research and scientific debate regarding long-term ecological impacts, and it has escaped experimental field trials in the western U.S. (Reichman et al. 2006; Watrud et al. 2004; Zapiola et al. 2008). *A. stolonifera* and some closely-related species are perennial weeds in the U.S. and other countries (Behrendt and Hanf 1979; MacBryde 2006). They are also considered to be invasive in some natural areas (Invasive Plant Atlas 2009; Levine 2000). Bentgrasses can be dispersed by seed and vegetative stolons, the latter being a trait common to many weedy and invasive plants (Otfinowski and
Intraspecific and interspecific pollen-mediated gene flow has been documented for *Agrostis* in experimental and natural conditions (reviewed in MacBryde 2006). *A. stolonifera* and the closely-related *A. gigantea* are able to hybridize and form persistent populations in roadsides (Hart et al. 2009). However, there is little information about their habitat types, plant community associations, or species co-occurrence in today’s natural and managed ecosystems.

*Panicum virgatum* is a native, warm-season, perennial grass most commonly associated with the tall grass prairies of North America (Weaver and Fitzpatrick 1934). However, in northeastern North America, the presettlement distribution pattern for switchgrass is believed to have been a narrow zone adjacent to the coastal salt marsh (Niering and Warren 1980). Switchgrass is commonly sub-divided into two broad ecotypes, Upland and Lowland. Lowland ecotypes are notable for their larger stature and are typically tetraploids, while Upland ecotypes are somewhat smaller and can be either tetraploid or octaploid (Martinez-Reyna and Vogel 2002; Porter 1966). Switchgrass is an outcrossing species with strong self-incompatibility, increasing the likelihood of intraspecific gene flow (J M Martinez-Reyna & Vogel, 2002). In the northeastern U.S., the *Panicum* genus includes native species growing in natural areas, native species established as weedy populations, and one non-native species that can be a crop or weed (*P. miliaceum*, Proso millet). As a cultivated plant, switchgrass ‘source identified’ cultivars have been used for livestock forage, wildlife habitat, prairie restoration, horticulture, and erosion control. In recent years, switchgrass has been promoted as a potential lignocellulosic feedstock for ethanol production (Wright and Turhollow 2010). Field test permits were issued for GE
*P. virgatum* (2008-2010) with increased biomass, decreased lignin, improved digestibility, improved nitrogen use efficiency, increased drought tolerance, or sterility (ISB 2010).

Our study was designed to address the following ecological questions in natural, cultural and agricultural landscapes: 1) What is the current distribution of *Agrostis* and *Panicum* species with respect to inland and coastal ecoregions, habitat types, and plant community assemblages in the northeastern U.S.? 2) Do species co-occur in plant communities such that interspecific pollen-mediated gene flow could be promoted?, and 3) Are management or disturbance events commonly associated with *Agrostis* and *Panicum* populations? In addition to increasing knowledge about plant ecology, answers to these questions could inform decisions about field testing and commercialization of novel *Agrostis* and *Panicum* genotypes and traits.

**Materials and Methods**

**Study Site, Botanical Surveys, and Habitat Definitions**

Field studies were conducted in 2009 and 2010 in two ecoregions: Lower New England Ecoregion (subecoregion Southern New England Coastal Hills and Plains, abbreviated CHP) and North Atlantic Coast Ecoregion (subecoregion Southern New England Coastal Lowland, abbreviated CL) (Metzler and Barrett 2006; TNC 2010) (Figure 1). The CHP transects were located within the western boundary of 72°32’15.1218” to the eastern
boundary of 71°48'8.1678" W and the northern boundary of 42°1'18.9948" to the southern boundary of 41°25'33.927" N. The CL transects along the Long Island Sound were located within the western boundary of 73°30'2.6202" to the eastern boundary of 71°27'24.3642" W and from the northern boundary of 41°26'3.9186" to the southern boundary of 41°02'37.0644" N.

Transect surveys (50 m) were conducted in late summer (July-September) and were replicated at twenty locations in each habitat type except for hayfields which were replicated 10 times due to low plant species diversity. The beginning and end points of each 50 m transect was recorded using a Juno SB handheld GPS (Trimble Navigation Limited, Westminster, CO USA). Tree canopy (shade) was measured along each transect at 10, 25, and 40 meters using a spherical densiometer (Model-C, Forest Densiometers, Bartlesville, OK) and then averaged. A 50 m measuring tape was laid on the ground and every vascular plant that fell directly above or below was identified and the number of ramets counted; this data was used for all calculations. In addition, target species (Agrostis, Panicum, and invasive plants) were recorded in a belt extending 1 m on each side of the transect line, while not used directly for any calculations, belt transect information provided additional detail for presence/absence purposes. Carex and Juncus species were classified to the genus level. Plant species nomenclature and nativity were as defined by the National PLANTS Database (2010). Invasive plants for the region were based upon a list from the Connecticut Invasive Plant Council (2009).
There were seven habitats in the CHP subecoregion: core forest, edge forest, herbaceous meadow, roadside, wasteland, hayfield, and cornfield. Six habitat types were defined based upon prior knowledge of plant communities. Core forest transects were used as a comparator because they are the closest available representation of undisturbed, climax vegetation in this region. Core forest was defined by a forest fragmentation map based on three criteria: 1) second growth forest, 2) at least 91.44 meters to any non-forested area in all directions, and 3) total forest area of 202.34 hectares or greater (Fig. 1) (CLEAR 2006). Twenty core forest transects were distributed across the CHP region, targeted transects representing the other six habitat types were clustered as close as possible to the core forest transects. Once the core forest points were chosen, the other transects were targeted based on the criteria listed below. Edge forest was defined as the transition between second growth forest and herbaceous plant communities and laid perpendicular to the transition. Herbaceous meadows were defined based on the dominance of herbaceous plants, while being maintained through mowing and suppression of woody plants (Metzler and Barrett 2006). Roadside transects were defined as locations within 2 meters along the margins of two-lane state highways. Wasteland transects were defined as areas with extensive, recent human management activities and/or disturbance (e.g. gravel parking lots and the edges of dirt roads). Hayfields were defined by the presence of crop species (e.g. orchard grass, smooth brome, timothy, and clovers) with mowing and harvesting 2-3 times per year. Cornfield edges (cornfield) were defined by the presence of Zea mays grown as an agronomic row crop. Transects ran along the edge of the cultivated field and included the last row of corn plants.
Transects representing three habitat types in the coastal CL subecoregion were difficult to establish because most of the coastal land is privately owned and disturbed. Transects were replicated in 20 locations for CL semi-natural, human impacted, and roadside (Fig. 1). The CL semi-natural habitat was defined as a coastal area with infrequent human traffic and where plant communities experienced minimal management. The CL human-impacted habitat was defined as a coastal area with frequent human traffic and often managed as part of a recreation area (e.g. beach, boat launch). The CL roadside habitat was defined by the same criteria as the CHP roadside habitat.

Plant communities can be defined as the collection of species at a specified place and time (Magurran 2004); in our study we defined a plant community as the taxa observed along a 50 m transect. Disturbance was defined as any relatively discrete event in time that disrupted the ecosystem, community, or population structure and changed resources, substrate availability, or the physical environment (White and Pickett 1985). Habitat was defined as an environment in which organisms live including the biotic and abiotic factors influencing the surroundings (Gurevitch et al. 2006).

Data Analysis
Records of the vascular plants and environmental variables observed in 190 transects were entered into Excel for data management (Microsoft Corporation, Redmond, WA). Species diversity was calculated using Shannon’s Diversity Index (SDI) because there were many singletons and SDI is sensitive to rare occurrences (Gurevitch et al. 2006). Two-way analysis of variance (transects were random effects, habitats were fixed effects)
was used to determine differences between SDI values (SAS ver. 9). Nonmetric multidimensional scaling (NMDS) was used to compare dissimilarities among plant communities (Kruskal 1964) and was computed using the Bray-Curtis method (Bray and Curtis 1957), R with the vegan package (R Development Core Team 2009, Oksanen et al. 2009), and plotted using Sigmaplot v10 (Systat, Chicago, IL). The statistical package R computed relationships between communities by calculating the closest representation on a two axis plot. The $r^2$ value was determined by calculating the correlation between actual dissimilarity and plotted dissimilarity (how well the plot configuration matches the data). Using the dissimilarities, we plotted the habitats in a hierarchical cluster analysis dendrogram using the single linkage method in the vegan package in R (Oksanen 2009). Branch length equaled “height”, the measure of similarity of the joined clusters.

Co-occurrence was computed for *A. stolonifera* and *P. virgatum* using the formula:

$$C_{ih} = 1 - 0.5 \sum |p_{ij} - p_{hj}|$$ (Schoener 1969, Silva and Batalha 2009). In this equation, $C$ is the co-occurrence value of the $i^{th}$ and $h^{th}$ species and $p$ is the proportion of occurrence of the $i^{th}$ or $h^{th}$ species in the $j^{th}$ community (transect). Our project compared habitats where plants differed in size. In order to compensate for size differences, we calculated the co-occurrence data using presence/absence information from transects instead of species abundance values.

Rarefaction curves are frequently used in order to estimate species richness based on measurements and determine if more measurements are needed. Rarefaction curves were computed in the program EstimateS (Colwell 2009). Plant species rarefaction curves
were plotted for twenty replicated transects in each habitat type (data not shown). The curves for hayfields and core forest habitats reached a plateau before the 20th replicate transect. Rarefaction curves for other habitats were beginning to reach an asymptote before the 20th replicate. While surveys of additional transects would have been ideal, it was not possible due to limited human resources, time, and access to some habitat types.

Results

Vascular Plants and Habitat Types

In the inland CHP region, 366 vascular plant species and 53,720 individuals were recorded in 130 transects conducted across seven habitat types (Table 1). The most abundant plant species across all transects was *Digitaria sanguinalis* (5,144 ramets) which was also the most common species (presence/absence) in CHP roadside habitats (Table 1). There were twenty singletons in 130 CHP transects. The seven CHP habitats were distinguished from each other by their plant communities and one environmental factor (canopy cover) (Table 1). Core forests had the second lowest values for SDI (7.08); only cultivated hayfields had less plant species diversity. Core forests had very few exotic or invasive plants and the most common species were native, consistent with our belief that these transects were the closest representation of undisturbed native vegetation (Table 1). Core forest transects had the most shade (86% canopy cover) and significantly more canopy cover than edge forest (65%) or other habitat types. Edge forest habitat had the highest SDI (Table 1). Even though the % invasive plant ramets
was higher than other habitat types (except for wasteland and hayfield), the value for % exotic (non-native) ramets was not significantly different to meadow, wasteland, or roadsides (Table 1). Similar to core forests, the five most common species were native plants. Plant diversity was relatively high for meadows, wastelands and roadsides but not significantly different from one another. In the wasteland, meadow, and roadside habitats, the exotic species, *Agrostis stolonifera*, was included in the five most common species. The habitats with the lowest plant diversity indices were cornfields and hayfields. The high % exotic plants were consistent with the knowledge that these were agricultural sites with intensive cultivation and weed management practices. The most common species included crops (e.g. *Zea mays*, *Trifolium purpurea*) and agricultural weeds (e.g. *Panicum dichotomiflorum*, *Digitaria*, *Plantago*). The relatively high % of invasive ramets in hayfields was due to one transect with many *Euphorbia cyparissias* ramets.

Transects (60) in the CL region near Long Island Sound contained 196 vascular plant species, 16,925 individuals, and 15 singletons. The most abundant species were *P. virgatum* (3,544 ramets) and *Digitaria sanguinalis* (1,132 ramets). The increased abundance of *P. virgatum* in the CL region compared to the inland CHP region was best explained by both actual increased abundance and an unintended effect of the targeted experimental design (transect sites were not random geographic points). Comparison of the chosen CL habitats did not show any difference in shade, species diversity, or abundance of exotic or invasive plants (Table 1). Weed species (e.g. *Digitaria sanguinalis*) and invasive species (e.g. *Celastrus orbiculatus*, *Phragmites australis*) were
among the most common plants, providing evidence of disturbance and/or degradation in the coastal plant communities studied.

A single-linkage dendrogram was created to represent the similarity/dissimilarity of habitat types based on observed plant taxa (Fig. 2). Hayfields and cornfields (agricultural systems) were most dissimilar to the natural or semi-managed habitat types. Core forests and edge forests showed similarity to each other, but dissimilarity to other habitats. Roadsides from the CHP and CL regions were closely related, as were CL semi-natural and human-impacted habitats. Other data supports the similarity of CL and CHP roadside habitats due to the non-significant difference between species diversity index, % invasive ramets, % exotic plant ramets, and most common plants (*Digitaria sanguinalis* and *Ambrosia artemisiifolia*) (Table 1). NMDS ordination plots for dissimilarity showed each transect as a discrete point and these points can be interpreted as observed plant communities (Fig. 3a-b). While no two plant communities were exactly the same, the ordination plot for CHP transects showed strong clustering of core forest and hayfield transects (Fig. 3a). Edge forest and cornfield transects also showed loose clustering. The ordination plot for the coastal CL region produced less clustering but showed a trend for roadside transects to group, but some roadside, semi-natural, and human impacted transects overlapped (Fig. 3b).

**Agrostis species**

Eight species in the bentgrass group were identified and receiving populations for intraspecific and interspecific gene flow were common (Table 2). Three species (*A.
hyemalis, A. perennans, and A. scabra) were native. The native A. perennans was unusual because it was the only Agrostis to occur in core forest habitats and tolerate higher amounts of shade (42% canopy cover compared to 0-18% for all other bentgrasses) (Table 2). Two members of the bentgrass group were not observed in this study (Polypogon viridis and Polypogon monspeliensis) although they have been included in regional floras (Dowhan 1979).

In both CL and CHP regions, transects with Agrostis species had a higher SDI than transects without Agrostis (Fig. 4). However, there were no significant differences in diversity values within each habitat type (data not shown). Agrostis species were more abundant in the CHP region (27.6 ramets/transect) than the CL region (12.2 ramets/transect). Agrostis species were most common in edge forest, meadow, roadside, and wasteland habitats which were also observed to have relatively high numbers of exotic and invasive plants (Table 1-2). Using a NMDS ordination plot, Agrostis species appear to be clustered in edge forest, herbaceous meadow, wasteland, and roadside habitats (Fig. 5a).

Attention was focused on A. stolonifera distribution and habitats because it has been proposed as a GE turfgrass. A. stolonifera was associated with four habitats: edge forest, herbaceous meadow, wasteland, and roadside (Fig. 5a). C values calculated for the inland region revealed three Agrostis species as among the top ten species for co-occurrence with A. stolonifera (Table 3). In the CL region, A. gigantea had a high C value (0.79) for
co-occurrence with *A. stolonifera* (Table 4). This provides evidence that bentgrasses occur together at a localized scale (Table 3-4).

*Panicum* species

Five native *Panicum* species were observed (Table 2). Three *Panicum* species (*P. miliaceum, P. philadelphicum, P. verrucosum*) were not observed in this study although they have been reported in regional floras (Barkworth et al. 2007). *Panicum amarum*, which is listed as threatened or of special concern in Connecticut and Rhode Island respectively, was only identified in five coastal CL transects in either semi-natural or human impacted habitats (Table 2).

In the inland CHP region, *Panicum* species were found in all habitat types except core forest. Roadsides, wasteland, and cornfields had the highest percentage of transects with *Panicum*. Fewer such transects were observed in edge forest, meadows, and hayfields (Fig. 5; Table 2). In the CHP region, *P. virgatum* was most frequently found in roadside habitat, but it was equally distributed across the three coastal CL habitats studied (Fig. 5c-d; Table 2). In contrast, *P. dichotomiflorum* was most abundant in CHP cornfield, wasteland, and roadside habitats, but less common in coastal habitat types (Table 2). *P. capillare* and *P. rigidulum* were found mainly in cornfields and herbaceous meadows, respectively. Unlike *Agrostis*, transects with *Panicum* had the same species diversity as transects without *Panicum* (Fig. 4a-b).
Analysis focused on P. virgatum distribution and habitats because it has been proposed as a GE biofuels feedstock crop. Receiving populations for intraspecific gene flow were observed, especially in roadside habitats in the CHP region (Fig. 5c) and in all habitats in the CL region where P. virgatum showed relatively even distribution (Fig. 5d; Table 2). Furthermore, a greater abundance of P. virgatum was observed in CL region than in the CHP region. Roadside habitats can be compared because of their similarity between regions; we found that P. virgatum had a median abundance of 42 ramets (n=9) in the CHP region and 84 (n=15) in the CL region. The association of P. virgatum and roadside habitat supports an observation by Haines (2010). P. virgatum was relatively uncommon in CHP herbaceous meadows (5%), a surprising result because this habitat could be thought of as the closest analogue to prairies (Barkworth et al. 2007). In the CHP region, 64% percent of transects with P. virgatum also contained one or more Panicum species; only 30% of transects with P. dichotomiflorum contained P. virgatum (data not shown). This indicates niche overlap, but C values showed low co-occurrence (Tables 3-4). Of the five transects with P. amarum, P. virgatum was only present in one, giving the two species a low co-occurrence value (-0.58) (Table 4).
Discussion

Patterns of grass cultivation in the U.S. can be expected to change due to land use and climate. For example, *P. virgatum* could be planted over large areas for biofuel production, and its suitable habitat could shift northward in this century due to climate change (Barney and DiTomaso 2010). *P. virgatum* continues to receive genetic modification through selection, breeding, and engineering to create novel agronomic traits, improve adaptability, increase stress resistance, and promote efficient biofuel production. These novel traits could expand both crop range and its cultivation in marginal lands. In the case of *A. stolonifera*, golf course construction, maintenance, and abandonment are major factors in distribution, as well as use of this species in drainage swales, lawns, and other constructed grasslands. Novel traits focus on improving turfgrass management and stress tolerance (e.g. herbicide resistance, salt tolerance). Concerns about future unintended consequences from pollen-mediated gene flow, the development of new weeds or invasives, and loss of genetic diversity in native grass populations have been expressed (Andow and Zwahlen 2006; Stewart et al. 2003; Wilkinson and Tepfer 2009). One response to these concerns is research leading to a detailed understanding of plant species distribution, communities, habitats, and species co-occurrence that will support ERA problem formulation, tiered risk assessment research, risk management, monitoring schemes, and exclusion zones (Wilkinson and Tepfer 2009).
We conducted field surveys to address risk assessment issues for *Agrostis* and *Panicum* grasses in the northeastern U.S. Analysis showed that the habitats studied in the inland CHP region were relatively distinct from each other with regard to plant communities, while the three coastal CL habitats overlapped (Table 1; Fig. 2-3). *Agrostis* and *Panicum* species were not equally distributed across all habitats and ecoregions (Table 2; Fig. 5a-d). The higher biodiversity of plant communities with *Agrostis stolonifera* may be due to the increased presence of exotic species. This biotic homogenization of the region is causing an increase of $\alpha$ diversity, especially in habitats that are managed by anthropogenic forces (Smart et al. 2006). Co-occurrence of exotic, weedy *Agrostis* species that can hybridize was common (Table 3). Overall, it was clear that receiving populations for pollen-mediated gene flow were common and distributed across the natural, cultural, and agricultural habitats studied. The observation of one *Panicum* species with special protection status raises questions about the possibility of interspecific gene flow leading to harm. However, there is little information about interspecific gene flow in *Panicum*, so the importance of co-occurrence for pollen-mediated gene flow is impossible to assess at this time.

Our results show that both *Agrostis* and *Panicum* species most commonly occur in habitats that have moderate to high human management and/or disturbance events. The diagram in Fig. 6 ranks habitat types along a continuum for disturbance and management. While somewhat subjective, this diagram promotes consideration of the likelihood and potential effects of management or disturbance events (e.g. herbicide application, winter deicing salt, irrigation) that could favor or discourage grasses with novel traits (e.g.
herbicide tolerance, salt tolerance, drought tolerance). This type of diagram could help design tiered risk assessment protocols for regulatory science. In our study, *Agrostis* species were most common in CHP habitats with intermediate management or disturbance regimes, while habitats at both ends of the spectrum (core forests, hayfields, cornfields) had few or no *Agrostis*. However, there was no difference in *Agrostis* between semi-natural, roadside, and human impacted habitats in the CL region. While *P. virgatum* was observed in semi-natural coastal habitat, it was similar to *Agrostis* in that it also occurred in disturbed or managed habitats (e.g. CHP roadsides, CL roadsides, CL human-impacted habitats) (Table 2, Fig. 5). This was somewhat surprising since *P. virgatum* is native to high salt marshes along the coast, a fact supported by observed differences in abundance. Studies of the invasive grass *Phragmites australis* showed that spread was facilitated by optimal growing conditions along roadsides and other rights-of-way (Jodoin et al 2008; Maheu-Giroux and de Blois 2007). These areas functioned as linear wetland networks with anthropogenic disturbance that assisted *Phragmites* dispersal. In our study, *Phragmites australis* was in the ‘top ten’ vascular plants most likely to co-occur with *P. virgatum* in the coastal CL region (Table 4). Thus, roadsides habitats could function as linear networks facilitating the spread of *P. virgatum, Phragmites*, and other species. Irrigation ditches, powerline rights-of-way, and railroad rights-of-way have also shown function as corridors for grass dispersal (Ahrens et al. 2011; Mallory-Smith and Zapiola 2008). The function of these linear corridors should be studied prior to the release of GE grasses because retraction of transgenes is nearly impossible and they might persist without ongoing selection pressure (Marvier and Van Acker 2005; Warwick et al. 2007; Zapiola et al. 2008). Information about dispersal mechanisms, in
combination with knowledge about plant community assemblages and plant species
distribution, will provide a strong basis for ecological risk assessments, predictions about
pollen-mediated gene flow, containment strategies, monitoring programs, and geographic
exclusion zones.

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Fig. 1 Location of transects (190) in ten habitat types in two ecoregions in the northeastern United States: Lower New England Ecoregion (subecoregion Southern New England Coastal Hills and Plains), and North Atlantic Coast Ecoregion (subecoregion Southern New England Coastal Lowland). Inland ecoregion transects were clustered near core forest habitat.
**Fig. 2** Dendrogram calculated from Bray's-Curtis (1957) dissimilarity, comparing all habitats in both subecoregions. Height is the dissimilarity distance. Abbreviations similar to Table 1 except for: CL Semi-natural, CL Nat; CL Human Impacted, CL HI.
Fig. 3 Non-Multidimensional Scaling (NMDS) ordination plots based on dissimilarities of transects (plant community assemblages) using Brays-Curtis analysis. Circles within the plots represent clustering for habitat types. a) CHP subecoregion with 130 transects ($r^2 = 0.70$), b) CL subecoregion with 60 transects ($r^2 = 0.69$).
Fig. 4 Comparison of Shannon’s Diversity Index for transects with and without Agrostis or Panicum species in two ecoregions. a) CHP ecoregion, b) CL ecoregion. Letters indicate significant difference at p < 0.05.
**Fig. 5** Non-Multidimensional Scaling (NMDS) ordination plots for two ecoregions based on dissimilarities of transects (plant community assemblages) using Brays-Curtis analysis. a) outlined symbols represent *Agrostis stolonifera* in the CHP region, b) outlined symbols represent *A. stolonifera* in the CL region, c) outlined symbols represent *Panicum virgatum* in the CHP region, and d) outlined symbols represent *P. virgatum* in the CL region. Values for $r^2$ as shown in Fig 3.
Fig. 6 Habitat types arranged according to degree of disturbance events or management activities. Habitats shown above the line were studied in the coastal CL region, habitats below the line were studied in the inland CHP region.
Table 1 Habitat types studied in the Lower New England ecoregion, Southern New England Coastal Hills and Plains (CHP) subecoregion, and the North Atlantic Coast ecoregion, Southern New England Coastal Lowlands (CL) sub-ecoregion. Letters indicate significant differences between habitats within each ecoregion ($\alpha = 0.05$). Abbreviations: number of transects observed ($n_t$), mean Shannon’s Diversity Index ($e^H$), mean % transect covered by tree or shrub canopy (% CC), mean % invasive plant species (# invasive ramets / # ramets in transect) (%inv), mean % exotic plant species (# non-native ramets / # ramets in transect) (%exotic), number plant species observed in habitat type (# species), mean number of plant species per transect (#species/transect), five most common species based on presence/absence in transects (Five most common species).

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<th>$e^H$</th>
<th>%CC</th>
<th>%inv</th>
<th>% exotic</th>
<th># species</th>
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Table 2  Bentgrasses (*Agrostis* and *Apera*) and *Panicum* species observed in habitat types in inland and coastal subecoregions. Values shown below each habitat type represent percent transects with the species. Mean % canopy cover (CC) was calculated for transects with the species.

<table>
<thead>
<tr>
<th>Grass species</th>
<th>Common name</th>
<th>% CC</th>
<th>CHP sub-ecoregion</th>
<th>CL sub-ecoregion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Latin name</td>
<td></td>
<td>Forest Edge Meadow Road Waste Corn Hay</td>
<td>Seminat Human Imp Road</td>
</tr>
<tr>
<td><em>A.stolonifera</em></td>
<td>creeping bentgrass</td>
<td>12</td>
<td>0 35 60 55 50 0 10</td>
<td>20 20 5</td>
</tr>
<tr>
<td><em>A.gigantea</em></td>
<td>redtop</td>
<td>16</td>
<td>0 35 55 35 50 0 0</td>
<td>15 30 5</td>
</tr>
<tr>
<td><em>A.canina</em></td>
<td>velvet</td>
<td>17</td>
<td>0 0 20 5 15 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td><em>A.capillaris</em></td>
<td>colonial bentgrass</td>
<td>6</td>
<td>0 5 20 15 15 0 0</td>
<td>15 15 20</td>
</tr>
<tr>
<td><em>A.perennans</em></td>
<td>upland</td>
<td>42</td>
<td>15 35 30 25 10 0 0</td>
<td>5 5 15</td>
</tr>
<tr>
<td><em>A.scabra</em></td>
<td>rough bentgrass</td>
<td>2</td>
<td>0 0 10 0 10 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td><em>A.hyemalis</em></td>
<td>winter bentgrass</td>
<td>18</td>
<td>0 5 5 5 30 0 0</td>
<td>5 0 10</td>
</tr>
<tr>
<td><em>Apera spica-venti</em></td>
<td>loose silkybent</td>
<td>0</td>
<td>0 0 0 5 0 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td><em>P.virgatum</em></td>
<td>switchgrass</td>
<td>1</td>
<td>0 0 5 45 15 0 10</td>
<td>65 70 75</td>
</tr>
<tr>
<td><em>P.rigidulum</em></td>
<td>redtop</td>
<td>0</td>
<td>0 0 15 0 0 0 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td><em>P.dichotomiflorum</em></td>
<td>fall panicgrass</td>
<td>9</td>
<td>0 5 10 50 60 95 0 5 5 30</td>
<td></td>
</tr>
<tr>
<td><em>P.capillare</em></td>
<td>witchgrass</td>
<td>0</td>
<td>0 0 0 0 5 15 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td><em>P.amarum</em></td>
<td>bitter</td>
<td>0</td>
<td>0 0 0 0 0 0 0 20 5 0</td>
<td>panicgrass</td>
</tr>
</tbody>
</table>
Table 3 Plant species that co-occur with *Agrostis stolonifera* and *Panicum virgatum* in the inland CHP subecoregion. The 10 species with highest C values for co-occurrence are shown, as well as five species least likely to co-occur.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>C value</th>
<th>Plant species</th>
<th>C value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agrostis stolonifera</em></td>
<td>1.00</td>
<td><em>Panicum virgatum</em></td>
<td>1.00</td>
</tr>
<tr>
<td><em>Agrostis gigantea</em></td>
<td>0.39</td>
<td><em>Poa trivialis</em></td>
<td>0.77</td>
</tr>
<tr>
<td><em>Symphorichum ericoides</em></td>
<td>0.37</td>
<td><em>Bromus tectorum</em></td>
<td>0.74</td>
</tr>
<tr>
<td><em>Potentilla simplex</em></td>
<td>0.35</td>
<td><em>Polygonum hydropiper</em></td>
<td>0.73</td>
</tr>
<tr>
<td><em>Juncus spp.</em></td>
<td>0.28</td>
<td><em>Polygonum careyi</em></td>
<td>0.73</td>
</tr>
<tr>
<td><em>Agrostis capillaris</em></td>
<td>0.26</td>
<td><em>Apera spica-venti</em></td>
<td>0.72</td>
</tr>
<tr>
<td><em>Agrostis canina</em></td>
<td>0.26</td>
<td><em>Anthemis arvensis</em></td>
<td>0.71</td>
</tr>
<tr>
<td><em>Dicanthelium sphaerocarpum</em></td>
<td>0.24</td>
<td><em>Eupatorium perfoliatum</em></td>
<td>0.71</td>
</tr>
<tr>
<td><em>Schizachyrium scoparium</em></td>
<td>0.24</td>
<td><em>Saponaria officinalis</em></td>
<td>0.71</td>
</tr>
<tr>
<td><em>Chaenorhinum minus</em></td>
<td>0.22</td>
<td><em>Medicago sativa</em></td>
<td>0.71</td>
</tr>
<tr>
<td><em>Setaria faberi</em></td>
<td>-0.47</td>
<td><em>Panicum dichotomiflorum</em></td>
<td>-0.14</td>
</tr>
<tr>
<td><em>Thelypteris noveboracensis</em></td>
<td>-0.48</td>
<td><em>Ambrosia artemisiifolia</em></td>
<td>-0.16</td>
</tr>
<tr>
<td><em>Quercus rubra</em></td>
<td>-0.51</td>
<td><em>Vaccinium corymbosum</em></td>
<td>-0.17</td>
</tr>
<tr>
<td><em>Vaccinium corymbosum</em></td>
<td>-0.54</td>
<td><em>Solidago rugosa</em></td>
<td>-0.30</td>
</tr>
<tr>
<td><em>Acer rubrum</em></td>
<td>-0.72</td>
<td><em>Acer rubrum</em></td>
<td>-0.60</td>
</tr>
</tbody>
</table>

# Listed as a weed in weeds of the northeast (Uva et al. 1997).
* Listed as an invasive plant by CT Invasive Plant Council (http://nbii-nin.ciesin.columbia.edu/ipane/ctcouncil/CT_invase.htm).
Δ Listed as an exotic species (USDA plants database, http://plants.usda.gov/).
Table 4 Plant species that co-occur with *Agrostis stolonifera* and *Panicum virgatum* in the coastal CL subecoregion. The 10 species with highest C values for co-occurrence are shown, as well as five species least likely to co-occur.

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>C Value</th>
<th>Plant Species</th>
<th>C Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agrostis stolonifera</em></td>
<td>1.00</td>
<td><em>Panicum virgatum</em></td>
<td>1.00</td>
</tr>
<tr>
<td><em>Rubus pensilvanicus</em></td>
<td>0.79</td>
<td><em>Celastrus orbiculatus</em></td>
<td>0.30</td>
</tr>
<tr>
<td><em>Agrostis gigantea</em></td>
<td>0.79</td>
<td><em>Toxicodendron radicans</em></td>
<td>0.22</td>
</tr>
<tr>
<td><em>Poa palustris</em></td>
<td>0.79</td>
<td><em>Euthamia graminifolia</em></td>
<td>0.21</td>
</tr>
<tr>
<td><em>Secale cereale</em></td>
<td>0.79</td>
<td><em>Ambrosia artemisiifolia</em></td>
<td>0.21</td>
</tr>
<tr>
<td><em>Sonchus oleraceus</em></td>
<td>0.79</td>
<td><em>Achillea millefolium</em></td>
<td>0.14</td>
</tr>
<tr>
<td><em>Amelanchier canadensis</em></td>
<td>0.79</td>
<td><em>Baccharis halimifolia</em></td>
<td>0.11</td>
</tr>
<tr>
<td><em>Boehmeria cylindrica</em></td>
<td>0.79</td>
<td><em>Digitaria sanguinalis</em></td>
<td>0.09</td>
</tr>
<tr>
<td><em>Agalinis tenuifolia</em></td>
<td>0.79</td>
<td><em>Schizachyrium scoparium</em></td>
<td>0.06</td>
</tr>
<tr>
<td><em>Lespedeza intermedia</em></td>
<td>0.79</td>
<td><em>Dicanthelium clandestinum</em></td>
<td>0.04</td>
</tr>
<tr>
<td><em>Vicia sativa</em></td>
<td>0.79</td>
<td><em>Phragmites australis</em></td>
<td>0.02</td>
</tr>
</tbody>
</table>

# Considered a weed in the northeastern U.S. (Uva et al. 1997).
* Considered an invasive plant in the region (http://nbii-nin.ciesin.columbia.edu/ipane/ctcouncil/CT_invasive.htm).
Δ Considered an exotic species (USDA Plants database, http://plants.usda.gov/).
Pollen longevity and dispersion models for switchgrass (*Panicum virgatum* L.)

(Presented in the format as published in Crop Science)

Ecological risk assessment (ERA); Genetically Engineered (GE); Pollen Half-life ($t_{1/2}$).

Abstract

Switchgrass (*Panicum virgatum* L.) is a North American grass that is being improved through conventional breeding and genetic engineering to create a biofuel feedstock. However, the introduction of novel traits has created concerns about pollen-mediated gene flow and negative environmental impacts. The objective of this study was to model switchgrass pollen dispersal using a Lagrangian approach informed by data on pollen longevity and size. With outdoor exposure, the viability of pollen from three cultivars declined over 60 minutes, but rare events showed pollen longevity of up to 100 minutes. To model pollen dispersal, wind fields were measured in two locations and on two dates to create case studies representing light wind conditions with buoyant turbulence, or stronger winds with pressure-driven, non-turbulent conditions. In the first case study, switchgrass pollen entrained in light wind conditions with buoyancy-driven turbulence moved up to ~3.5 km from the source with a maximum flight time of 6000 s. In the second case study, pollen released in stronger winds with pressure-driven
conditions moved up to ~6.5 km with a maximum flight time of approximately 1300 s. In both cases, the majority of pollen grains were deposited closer to the source. These case studies provide information helpful for predicting pollen-mediated transgene flow, isolating field trials, creating containment and co-existence strategies, and conserving valued switchgrass populations in coastal areas and prairies.

Switchgrass (*Panicum virgatum*) is a North American, rhizomatous perennial that is expected to have many advantages as a biofuel crop including high yield and growth on marginal lands (Heaton et al., 2008; Jakob et al., 2009; Parrish and Fike, 2005, Schmer et al., 2008; Jung and Vogel, 2001; Wright and Turhollow, 2010). The quest for renewable energy has led to switchgrass improvement programs utilizing selection of native genotypes, hybridization, molecular markers and genetic engineering (Bouton, 2007; Casler et al., 2007; Heaton et al., 2008; Jung and Vogel, 2001, Kwit and Stewart, 2012; Stewart, 2007; Taliaferro, 2002). However, the introduction of novel traits to a widely distributed, native grass has raised concern among switchgrass breeders, seed producers, farmers, land managers, regulators and ecologists. These concerns have primarily focused on crop-to-wild gene flow, increased weediness, and ecological impacts (Kwit and Stewart, 2012; Kausch et al., 2010).

An ecological risk is defined as the co-occurrence of a hazard (adverse environmental impact) and an exposure (mechanism/route by which the hazard is experienced) (Andow and Zwahlen, 2006; Auer, 2008; Wolt, 2009). Potential hazards from biofuel crops and their novel traits can be grouped into two broad categories: 1) gene flow leading to the loss of unique genotypes in native populations or creating new weeds, and 2) direct and indirect effects on
habitats and non-target organisms (Barney and DiTomaso, 2008; DiTomaso et al., 2007; Raghu et al., 2006; Wolt, 2009). One major concern for switchgrass as a biofuel feedstock is that native genotypes will be changed or lost after large areas are planted with improved cultivars or genetically engineered (GE) plants (Casler et al., 2007; Kwit and Stewart, 2012). Native switchgrasses can be separated into two broad ecotypes as well as genetically-distinct regional populations across most of North America (Casler et al., 2007; Morris et al., 2011; Zalapa et al., 2011). Switchgrass has many characteristics associated with the high levels of gene flow observed in other grasses including minimal domestication, out-crossing and self-incompatibility (Martínez-Reyna and Vogel, 2002; Zalapa et al., 2011). A study in the Northeastern US showed that switchgrass populations (native, feral, and cultivated) were associated with specific habitats, and these plants could be receiving populations for crop-to-wild gene flow (Ahrens et al., 2011). In addition to gene flow, there are concerns about increased weediness or invasion because switchgrass has not undergone extensive domestication and has weedy traits such as rapid growth, tolerance of a wide range of environmental conditions (e.g. salt exposure), large numbers of pollen grains and seeds released over a long time period, dispersal by rhizomes, and successful competition in diverse communities outside of cultivation (Barney and DiTomaso, 2008; Parrish and Fike, 2005). Additional novel traits that increase plant fitness could exacerbate the situation (Barney and DiTomaso, 2008; Raghu et al., 2006). Several closely-related Panicum species are economically-important weeds (e.g. P. capillare, P. dichotomiflorum) (Uva et al., 1997). Some farmers already consider switchgrass a potential weed and, in at least one instance, have requested that it not be planted beside their fields (James et al., 2010).

Vertical gene flow occurs when viable hybrid offspring are produced between sexually-compatible plants, and new genes are stabilized (introgressed) in the genome of the recipient
plant population (Auer, 2008). Many discussions about switchgrass gene flow have focused on the dispersion of viable pollen, a single step within gene flow, because regulators and other stakeholders need to know distances that will provide containment of experimental field trials or co-existence among producers (Kausch et al., 2010; Kwit and Stewart, 2011; Stewart, 2007). The escape of GE creeping bentgrass in Oregon has also increased interest in predictive models for pollen dispersal (Charles, 2011; Watrud et al., 2004; Pfender et al., 2007). Unlike air pollution, switchgrass pollen dispersion is best modeled using an advective particle transport approach because pollen grains are too large to be treated as one chemical suspended in another (Sawford & Guest, 1990). Switchgrass pollen dispersion models require an understanding of the wind field, the pollen’s motion in the wind field, and termination of the pollen flight due to loss of viability or meeting an obstacle. There are two methods for modeling the wind field component, the Eulerian approach and the Lagrangian method. The Lagrangian method has been used by other researchers because the Eulerian approach has significant drawbacks and challenges: Eulerian models solve boundary-value partial differential equations that are impractical to parameterize for a particular site at a particular time. To do so requires full knowledge of the atmosphere everywhere on the control volume. Also, the equations are computationally and mathematically difficult to solve; supercomputers and highly specialized staff are required (Sawford & Guest, 1990). The Lagrangian model is based on Reynolds’s averaging (Stull, 2003), which models the wind field as mean flow components plus turbulence components. One advantage is that the deployment of anemometers yields a time series of wind speed and temperature data, all of the information needed to determine the relevant statistics. The Lagrangian approach has been used to model pollen dispersal in maize (Wang and Yang, 2009; Aylor et al., 2006; Arritt et al., 2007) and creeping bentgrass (Pfender et al., 2007).
Along with wind-field data, pollen dispersal models must be informed by factors such as pollen longevity, size and settling velocity. After anthesis, switchgrass pollen will be entrained in the wind until either coming to rest or losing viability in flight. Pollen longevity has been determined by testing the ability of pollen grains to produce seeds, in vivo germination on conspecific stigmas, in vitro germination on media, or vital stains (Dafni and Firmage, 2000). Pollen longevity has been characterized for various grasses including switchgrass (Ge et al., 2011; Bragg, 1969), corn (Zea mays) (Aylor, 2003, 2004; Fonseca and Westgate, 2005), creeping bentgrass (Agrostis stolonifera) (Fei and Nelson, 2003), tall fescue (Festuca arundinacea) (Wang et al., 2004), zoysiagrass (Zoysia japonica) (Kang et al., 2009), and Johnsongrass (Sorghum Halepense) (Burke et al., 2007). The viability of switchgrass pollen is affected by environmental variables such as humidity, UV radiation and temperature (Ge et al., 2011).

Settling velocity is another factor for models because pollen grains have a downwards component to their motion even if the air is carrying them upwards. Settling velocity is calculated using Stoke’s law and variables including pollen diameter and mass (Sosnoskie et al., 2009). Stoke’s law pertains to spheres moving through a fluid column under gravity attraction alone. We assume grass pollen grains are spherical, which implies they are not yet desiccated.

The objective of this study was to characterize specific switchgrass pollen traits and use that information in modeling pollen dispersion through a Lagrangian approach. To the authors knowledge, this is the first study that applies a Lagrangian approach to modeling switchgrass pollen dispersal. Pollen viability over time and pollen size were measured in common switchgrass cultivars. Sonic anemometers were used to measure wind fields at two discrete locations and times, allowing us to generate two pollen dispersal models as contrasting case studies (light wind with buoyant turbulence vs. stronger, pressure-driven, non-turbulent winds).
previous study with corn relied on only one location (Wang and Yang, 2010). Our case studies on switchgrass pollen dispersal help address one critical step in predicting crop-to-wild gene flow and population genetics. The results could be useful in isolating experimental field trials, maintaining seed identity, creating containment or co-existence strategies, or protecting valued switchgrass populations in coastal areas and prairies.

**Materials and Methods**

**Pollen Germination**

Three cultivars of *Panicum virgatum* were grown at the University of Connecticut research farm as pollen donors: ‘Blackwell’, an upland octoploid cultivar (Sharp Bros. Seed Co., Clinton, Missouri, USA); ‘Alamo’, a lowland tetraploid cultivar, (Sharp Bros. Seed Co., Clinton, Missouri, USA); ‘Kanlow’ a tetraploid lowland cultivar (USDA-GRIN, PI 421521, Kansas, USA). Pollen donors were grown in Fafard® Nursery Potting Mix (Conrad Fafard Inc., Agawam, MA, USA), fertilized with 3g of Nutricote® 18-6-8, type 180 (Arysta LifeScience, Chuo-ku, Tokyo, Japan) and grown under natural daylight conditions in a greenhouse (temperatures 19-38°C, mean=26°C).

The pollen germination media (PGM) was optimized using ‘Blackwell’ pollen harvested August-September 2011. Pollen was harvested from a single floret for each replicate Petri plate (Falcon® Ten-twenty-nine™ Petri Dish, Becton Dickson and Co., Franklin Lakes, New Jersey, USA). Newly dehisced anthers were observed from 11:00 to 13:30 each day. Experiments to optimize the PGM were based on published procedures for other grass species (Fei and Nelson, 2003; Wang et al., 2004) and contained 2% agar, 1 mM boric acid and 1 mM calcium nitrate (all chemicals from Sigma-Aldrich Corp., St. Louis, Missouri, USA) with sucrose concentrations at
1.0 M, 0.8 M, 0.6 M, or 0.4 M. Percent pollen germination was determined using a light microscope and five replicate plates in each treatment group. A 3 × 3 × 3 factorial in completely random design was used to further optimize the media using sucrose (0.5, 0.6, or 0.7 M), boric acid (1, 2, or 4 mM) and calcium nitrate (1, 2, or 4 mM).

Pollen viability experiments had four replicates of each exposure duration per day for each of the 7 days of exposure (n=28) (August-September 2010). The experiments were conducted on days with approximately 24°C, 53% RH, and light intensity 583 watts/m² (NOAA substation #06-8138-2, University of Connecticut Research Farm). Switchgrass pollen was transferred from florets to petri plate lids and moved outdoors for 0, 20, 40, 60, 80, or 100 min exposure to sunlight and ambient outdoor conditions. After exposure, pollen was immediately transferred to PGM media containing 0.5 M sucrose, 2 mM boric acid, and 1 mM calcium nitrate. Pollen was incubated in the dark at 25-30°C for one hour and then stored at 5°C until observed using light microscopy. Percentage pollen germination was based on observation of 200 pollen grains in each plate. Pollen was considered germinated if the length of the pollen tube was greater than the width of the pollen grain. Pollen half-life (t\textsubscript{1/2}) was calculated using the best fit exponential decay equation. Pollen of the upland, octoploid cultivar ‘Blackwell’ was measured (n=35) using light microscopy to determine diameter and calculate settling velocity.

**Switchgrass Anthesis**

The anthesis of 50 switchgrass panicles was studied in a wildlife management area (Sugarbrook Field Trial Area, Plainfield, CT, USA, GPS coordinates 41.716782, -71.940591) planted with a mix of native grasses including the switchgrass cultivar ‘Blackwell’. Fifty random points were generated and the switchgrass plants closest to each point were identified. One panicle from each plant was tagged and monitored during the flowering period (July-August
At each time point, panicles were classified based on cumulative anthesis (0%, 1-25%, 26-50%, 51-75%, 76-99%, 100%).

Pollen Dispersion Modeling

The pollen dispersion case studies utilized sonic anemometer data from two dates and locations. On 13 Aug 2010, wind data was collected from the wildlife management area (Sugarbrook Field Trial Area, Plainfield, CT, USA, GPS coordinates 41.716782, -71.940591). This date corresponded with the peak of switchgrass flowering. Sonic anemometers (Applied Technologies, Inc., Colorado, USA) had three axes and a 10 Hz sampling rate producing time-stamped data series with temperature (C) and wind speeds (m/s) in the x-, y-, and z-directions. These winds were dominated by buoyant turbulence with light mean wind speeds (data not shown). Wind data was also collected on 1 Sept 2002 at the University of Connecticut research farm in a corn field (GPS coordinates 41.795208, -72.228738) (Wang and Yang, 2010); this date corresponds to the latter half of the typical switchgrass flowering period. These winds were dominated by pressure-driven, non-turbulent winds (data not shown). Stokes’s law was used to calculate pollen settling velocity as follows: \( a = \text{grain’s radius (meters), } (20 \times 10^{-6} \text{ m}) \); density of fresh pollen= \( \rho_s (1410 \frac{kg}{m^3}) \); \( g = \text{gravity, } (9.81 \text{ m/s}^2) \); \( \gamma_s = \text{specific weight of a pollen grain } \gamma_s = g \rho_s \)

\( = (13832.1 \frac{N}{m^3}) \); \( \gamma = \text{specific weight of air, } (11.43 \text{ N/m}^3 \text{ and } 30^\circ \text{ C}) \); \( \mu = \text{dynamic viscosity of air, } (1.98 \times 10^{-5} \text{ N s/m}^2) \) (Aylor., 2002; Sosnoskie et al., 2009).

Mean wind speed was found using spectral analysis to determine the atmospheric waves having periods longer than 15 minutes by computing discrete Fourier coefficients for the 30-
minute, one-, two-, four-, and eight-hour harmonics for each wind component \((u, v, w)\), computing the inverse transformations, and computing the series’ trends. This effectively implemented a low-pass filtering of the wind field. These relatively long wavelength components above the spectral gap form the non-turbulent wind field. (Howell and Mahrt, 1996). The mean wind was subtracted from the observed time series observations yielding instantaneous fluctuations from the mean. These were partitioned into 15-minute blocks with the following flow parameters computed per block: potential temperature, standard deviation of the wind-speed components, friction velocity, Obokov length (Stull, 2003), and the Lagrangian time-scale coefficient (Wilson and Shum, 1992).

The Lagrangian model follows Wang and Yang (2010) and is a Markov process (Brownian motion or random walk) parameterized by the turbulence statistics (Aylor and Flesch, 2001; Sawford and Guest, 1991; Wilson and Shum, 1992). The window of time was 1100 h to 1330 h to reflect the time of anthesis. The Lagrangian model uses Taylor’s assumption (Stull 2003), which allows the wind field sampled at one point to describe the wind field everywhere else. The mental model is that all space is flat and covered homogeneously with switchgrass. In the model, a single pollen source at the origin releases 10,000 pollen grains in each 15-minute block. The grains are released throughout the time block in steps equal to the Lagrangian time-scale coefficient, starting over at the beginning as necessary to release all 10,000 grains. Flights were rejected if a grain reached an altitude of 100 m because boundary-layer processes begin to be replaced by general circulation models. Grains were considered to have fallen to the ground if: 1) a grain’s altitude fell below 1.5 m because it would have fallen into the switchgrass field where the wind field described by the Lagrangian model no longer pertains, or 2) the grain died in flight due to its natural longevity. All grains were allowed to live 100 min, so the dispersion
patterns are “worst case” in the sense that they allow all grains to live to the maximum time observed in our experiments. Points in the dispersal figures show the position where a simulated grain terminated its flight for either reason.

**Results and Discussion**

**Pollen longevity, anthesis, and size**

A factorial experiment to optimize PGM showed that pollen germination reached a statistically significant maximum (75%) on media containing 0.5 M sucrose, 1 mM calcium nitrate, and 2 mM boric acid. Pollen from all three switchgrass cultivars lost viability over approximately 60 min of exposure (Fig.1). Switchgrass ‘Blackwell’ pollen went from 74% to 5% germination after 60 min of exposure and reach 0% at 80 min; ‘Kanlow’ pollen diminished from 68% to 0.9% in 60 min, although one petri plate had 0.23% pollen surviving at 100 min; ‘Alamo’ pollen went from 76% to 0.03% in 60 min and reached 0% at 80 min. The exponential equation \( y = 95.6 e^{-0.056t} \) best fit the data \( (r^2=0.29) \). This low \( r^2 \) value reflects natural variation among cultivars and expected variability due to the handling of short lived pollen. Using this best fit equation, the predicted half-life of released pollen was \( t_{1/2}=17.3 \) min. Studies on switchgrass and other grass species show that most grass pollen loses viability in about 60 min (Fei and Nelson, 2003; Ge et al., 2011; Kang et al., 2009; Wang et al., 2004). This is consistent with anemophilous species and species where pollen is released frequently (Dafni and Firmage, 2000). Switchgrass panicles released pollen from late July through August (Fig.2). Anthesis reached its peak on August 10 when 84% of monitored panicles were actively releasing pollen (Fig.2).
Pollen of the upland, octoploid cultivar ‘Blackwell’ was measured using light microscopy to determine pollen diameter and calculate settling velocity. Pollen grains had a mean diameter of 43.7 µm (±2.9) with a range of 37.5-50.0 µm (n=35). This was slightly smaller than previously reported for upland, octoploid cultivars by Ge et al. (2011) (mean 47.2 µm, range of 43.0-51.0 µm), and slightly larger than reported by Bragg (1969) (mean 36.02 µm, range of 28.7-42.7 µm).

Model of Pollen Dispersal

Our Lagrangian models of switchgrass pollen dispersion were based on 100 min pollen viability, pollen settling velocity of 0.0638 m/s, and two wind field data sets from 1100-1330. Parameters used in this simulation, while appropriate for modeling switchgrass pollen dispersion, are generally relevant to other anemophilous grass species. In the first case study, the wind field from the wildlife management site suggested viable switchgrass pollen dispersal as far as ~3.5 km and in all directions (Fig. 3a). This case study represented relatively mild, summer wind conditions (0.09 m/s) where buoyancy-driven turbulence dominated. Pollen was scattered in all directions, even against the mean wind direction. This is possible because, on light wind days, solar ground heating creates winds whose horizontal speeds can exceed the non-turbulent winds but whose dominate direction is upwards, having no dominate horizontal wind direction are greater than non-turbulent wind speeds. The turbulent wind conditions resulted in modeling output with maximal flight times of 6,000 s, the longest flights allowed by the longevity component of the model (Fig. 3b). As expected, pollen deposition, as measured by modeled low pass filtering, decreased over distance from the source (Fig. 3c).

In the second case study, the wind field from the research farm resulted in a model suggesting that viable switchgrass pollen might travel as far ~6.5 km (Fig. 4a). This case study
represented a situation in which a storm front provided higher, non-turbulent average wind speeds (7.6 m/s). This scenario included gusty wind conditions, but these are not uncommon. The dispersion pattern was dominated by the mean wind direction, but the random walk creates spreading and produces a dispersion field akin to a Gaussian plume. The dominance of non-turbulent wind gusts resulted in maximal flight times of ~1300 s, significantly lower than those of the first case study (Fig. 3b). Pollen deposition decreased over distance, but was approximately twice the distance modeled for the first case study (Fig. 3c).

The maximum distances traveled by switchgrass pollen in these models are relevant, but for only a small proportion of the pollen grains released by a switchgrass field. The majority of pollen grains will lose viability in less than 100 minutes (Fig.1) or settle close to the release site (Fig. 3 and 4). A study based on observed long-distance pollen-mediated gene flow from creeping bentgrass fields showed that viable pollen moved at least 21 km in eastern Oregon (Watrud et al., 2004). Our case studies suggest distances less than that of creeping bentgrass, but the model does not account for the significant possibility of viable pollen being lofted by turbulent convection into the troposphere where the geostrophic winds are typically several times faster than boundary layer winds.

The estimates of pollen dispersion in this study are the first step in providing regulators and other stakeholders with distances useful for planning the containment of experimental field trials, identity preservation during seed production, segregation of switchgrass crops and the protection of remnant native switchgrass populations in coastal areas and prairies. Future models should represent a greater diversity of wind field data including higher atmospheric levels. In addition, more sophisticated models should include variables such as the decay in pollen longevity, landscape characteristics and geography.
Acknowledgements

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References


Figure 1. Longevity of switchgrass pollen from three cultivars exposed to outdoor conditions. Mean and standard deviation are shown for each time period (n=28).
Figure 2. Pattern of anthesis in switchgrass panicles. Panicles (n=50) were categorized by the percent florets which had undergone anthesis. Observations were made July-August 2010 at a wildlife management site (Plainfield, CT, USA) planted with a grassland mix including ‘Blackwell’ switchgrass.
Figure 3. Case study 1: Switchgrass pollen dispersion model based on a buoyancy-driven, turbulent wind field recorded August 2010 (Plainfield, CT, USA). a) Pattern of pollen dispersal with points showing the position where a simulated grain terminated its flight, b) Histogram representing the number of simulated pollen grains landing or losing viability over approximately 1300 s flight time. c) Histogram representing the distance traveled by simulated pollen grains.
Figure 4. Case study 2: Switchgrass pollen dispersion model based on a gusty, non-turbulent, pressure-driven wind field recorded September 2007 (Mansfield, CT, USA). a) Pattern of pollen dispersal with points showing the position where a simulated grain terminated its flight, b) Histogram representing the number of simulated pollen grains landing or losing viability over 6000 s flight time. c) Histogram representing distance traveled by simulated pollen grains.
Chapter 4

Sexual Compatibility of Switchgrass (*Panicum virgatum* L.) with the *Panicum* species of

Northeastern North America
Abstract

The characterization of inter-specific gene flow is an important part of ERA. When inter-specific gene flow is possible, the effects of a proposed transgene must be evaluated, not only in relation to transgenic crop itself, but also in relation to other compatible species. Because species level taxonomic divisions are not always a good indicator of two species ability to produce hybrid progeny, it is important to test congeneric species for compatibility when considering the release of a new transgenic variety. This project attempted to assess the inter-specific compatibility of Switchgrass with four other Panicum species. Panicles were bagged together and the seed yield from switchgrass was assessed. However, technical challenges prevented robust analysis of interspecific sexual compatibility.
Introduction

The characterization of interspecific gene flow is an important part of ecological risk assessment (ERA). When interspecific gene flow is possible, the effects of a proposed transgene must be evaluated, not only in relation to transgenic crop itself, but also in other sexually compatible species (B.-R. Lu & Yang, 2009). Because species level taxa are not always a good indicator of the ability to produce hybrid progeny, it is important to test congeneric species for compatibility when considering the release of a new GE crop. For example floras indicate that *P. virgatum* and *P. amarum* intergrade when found together (Barkworth, et al., 2008; Palmer, 1972). This suggests the possibility of gene flow, and transgene escape. Recent phylogenetic work also suggests that *P. amarum* is the *Panicum* species most closely related to switchgrass (Huang et al., 2011). In addition, there is at least one personal account that claims the two species hybridize in common garden experiments (Palmer, 1972). However, to this author’s knowledge, no formal study has been published demonstrating that the two species are sexually compatible.

In a similar manner, it is important to eliminate the potential for switchgrass to hybridize with *P. capillare*, *P. dichotomiflorum*, and *P. miliaceum* as these *Panicum* species are agricultural weeds (Colosi & Schaal, 1997; Uva et al, 1997). In addition, in recent years members of the genus *Dichanthelium* genus have been separated from *Panicum* (Barkworth et al., 2007). For this reason, one of the early objectives of this project was to learn whether it was likely that *Dichanthliem* species could be sexually compatible with switchgrass. It was determined that hybridization between switchgrass and *Dichanthliem* was unlikely in natural settings so that this should not be the focus of forced hybridization experiments. Two factors supported this decision based on two factors. First, the majority of *Dichanthliem* species are diploid, while switchgrass is predominantly either tetraploid or octoploid (Gould & Clark, 1978), and second, the two genera
rarely, if ever, release pollen concurrently (Figure 1). This study was designed to address the following questions: 1) Can switchgrass produce hybrid seed when crossed with congeneric species? 2) Is the resulting hybrid seed viable?

Materials and Methods

The Panicum and Dichanthelium species tested for compatibility with switchgrass included: *P. amarum, P. anceps, P. miliaceum, P. capillare, P. dichotomiflorum, D. meridionale, D. commutatum*. Taxonomy follows the treatment in Barkworth et al., (2007). The germplasm for *P. amarum, P. anceps, D. meridionale, and D. commutatum* was obtained from the USDA National Genetic Resources Program (GRIN), *P. amarum* seed was obtained from GRIN and field collected (Hebron, Connecticut). *P. miliaceum* seed was obtained from commercial bird seed mix (Blue Seal Feeds, NH). Seed for both *P. capillare and P. dichotomiflorum* was field collected (Storrs, CT). Plants were grown and panicles bagged in greenhouse conditions from March-September, 2010. Compatibility was tested by bagging panicles from two different species together as described in Martinez-Reyna & Vogel (1998). Plants were monitored first for anthesis and then confined to sealed glassine bags (Uline, Inc., Waukegan, IL). Florets which had already extruded anthers were removed. Co-bagged panicles were allowed to mature for 30 to 60 days before being removed and the switchgrass panicle (as female parent) was examined for the presence of mature caryopses. Putative hybrid seed was tested for viability in a greenhouse mist chamber. Viable progeny were tested to verify their parentage using microsatellite markers developed by Zalapa et al., (2011). For a detailed account of DNA preparation and analysis (see Chapter 4).
Representatives of each species were also grown in the summer of 2009 on drip irrigation at the University of Connecticut Plant Science Research and Education Facility (Figures 2-8). Anatomical features were measured, including height, blade length, and width. Counts were made of the number of shoots, panicles, and spikelets per plant. Estimates of total seed production were produced by multiplying the mean number of spikelets per panicle by the total number of panicles per plant.

Results and Discussion

Flowering Periods

One of the early objectives of this study was determining the sexual compatibility of switchgrass with other species by bagging panicles together. This was initially viewed as a simple procedure. Because crosses required only that the individuals reach anthesis at the same time. However, attempting the procedure on two different species proved far more complicated. One of the first hurdles to overcome in interspecies crosses was controlling flowering time. The Panicum species included in this study share a common general flowering period in this region from July to early October (Barkworth et al., 2007). However, all of the successful crosses in this project were performed in greenhouse conditions as outdoor moisture conditions damaged the glassine bags used in this study. The perennial Panicum species in this project (P. virgatum, P. amarum, and P. anceps) underwent biannual flowering when grown year round in greenhouse conditions. This allowed crosses with these species to be attempted twice per year; from March to May and July to September. This is consistent with previous switchgrass studies indicating that it has a facultative short-day response to photo-period (Van Esbroeck et al., 2003). Not surprisingly, the annual species were capable of just one robust period of flowering each growing
season. Two of these, *P. capillare* and *P. dichotomiflorum*, appeared to be sensitive to short day photoperiods; only they produced flowers in the late summer and early fall. *P. miliaceum*, however, behaved in a day-neutral manner flowering at maturity regardless of season. Although complex, it is possible to orchestrate other species to flower at the same time as switchgrass.

**Pollen Viability and Anthesis**

The challenge of timing was further complicated by the short-lived nature of switchgrass pollen, and presumably, that of the other *Panicum* species as well (Ecker et al., 2012; Ge et al., 2011). Unlike species in which pollen remains viable for days or weeks, or can be stored for extended periods (e.g. tomato; Song & Tachibana, 2007), switchgrass pollen survives for only around 60 min when harvested fresh at anthesis (see Chapter 2). In addition, all of the *Panicum* species in this project have asynchronous anthesis among the florets on a panicle. Switchgrass florets, for instance, go into anthesis starting at the top and outermost florets anthesis then proceeds down and inward on the panicle. This can be useful in that the opening of the uppermost florets serves a signal that other florets on the same panicle will soon reach anthesis and extend receptive stigmas. That said, floret emergence pattern varies among the species in this study and caused difficulties when bagging receptive florets of two different species together. The pattern in *P. capillare* is particularly difficult as florets reach anthesis as soon as they exit the leaf sheath and well before the structure of the whole panicle has fully expanded or matured. *P. miliaceum* produced panicles in which the florets were almost entirely synchronized and the flowering period only lasted one or two days. This may be a reflection of the fact that a commercial cultivar was used. The behavior of weedy genotypes may be different. *P. amarum*, *P. anceps*, and *P. dichotomiflorum* all exhibited floret maturation patterns similar to that of switchgrass.
Panicle Size and Growth Rate

Another challenge in this project was the size and growth rate of panicles, and associated plant parts, in the different species. Switchgrass is a large plant, up to 3m in height producing panicles that can be as large as 55cm long and 20cm wide (Barkworth et al., 2007). While stature was not a significant problem for crosses with P. amarum, differences in overall size with the other species were problematic. P. anceps, P. capillare, and P. dichotomiflorum all grow to maximum heights of between 1.3-2m making them one half to two thirds the size of switchgrass (Figure 2). This makes bringing the species flowering panicles together difficult because the peduncles of these grasses are rigid and prone to breaking when bent. Therefore, some crosses required small platforms to raise the height of smaller plants to that of switchgrass.

Another problem was that the point in time at which the florets on a panicle began to mature, when trimming and bagging must take place, the structure of the panicle itself is not yet mature. In fact, the panicles of these species were, still expanding in both length and width. In addition, the peduncles of these immature panicles were still growing and at rates that differed by age and species. Thus, the glassine bags protecting the two panicles often burst.

Morphological Measurements and Seed Set Estimates

Although the morphological measurements did not prove necessary for distinguishing putative hybrids, they are presented here as part of the scientific record. The heights, lengths, and widths of the leaf blades of all eight species fell within the ranges of treatments currently found in the literature (Figures 2-4). Notably, P. capillare, and P. dichotomiflorum grew much larger than in the ruderal environments in which I have generally observed them; this was likely due to the high nutrient and moisture conditions of the drip irrigation system. The species in this study produced an average of 15 to 40 shoots per plant (Figure 5). Only D. oligosanthes produced
substantially more with well over 100 shoots per plant. Finally, the mean number of panicles per plant and spikelets per panicle were used to estimate maximum seed yield if all flowers were fertilized, a likely outcome since a number of the species are self fertile (Figures 6-8). Not surprisingly, *P. capillare* and *P. dichotomiflorum* were by far the most fecund with an estimate of over 200,000 seeds per plant (Figure 8). This is consistent with observation that they are highly morphologically plastic and economically important weeds (Uva et al., 1997). In contrast, *P. miliaceum* produced many fewer seeds than its weedy counterparts despite also being a commercially important weed. This may reflect natural differences between species or it could be because the seed is from a commercial cultivar rather than a weedy genotype (Colosi & Schaal, 1997).

**Detection of Putative Hybrid Seed**

This project failed to produce evidence of interspecific hybridization between switchgrass and six other species. This is partly due to the numerous complications with forced hybridization methods discussed above. The failure to produce hybrids between switchgrass cultivar ‘Blackwell’ (8x) and *P. amarum* (4x) was likely due to mismatches in ploidy (Triplett et al., 2012; Zalapa et al., 2011) (Table 1).

One hybrid cross in this project did produce putative hybrid seed. Seed was produced between a GRIN switchgrass accession from North Carolina and *P. amarum* (Table 1). However, the validity of identification of the *P. amarum* parent was called into question as it came from a local CT inland site which contained a population with individuals similar morphologically to *P. virgatum* (open panicles) and *P. amarum* (compressed panicles). Seeds produced by this cross were viable and produced putative hybrid plants. DNA was extracted from parents and progeny, and 18 SSR markers were applied, and it was determined that the putative hybrid offspring were
in fact from the expected (data not shown). Unfortunately, subsequent attempts to replicate this finding with other lines of *P. amarum* parent were unsuccessful. Two GRIN accessions were grown to maturity, but they were not morphologically consistent with description of *P. amarum*.

**Future Directions**

This project was unable to produce a credible test of the sexual compatibility between switchgrass and other members of the *Panicum* genus. Future studies should testing compatibility from other species by exposing individual switchgrass florets to fresh pollen. This approach, while more labor intensive, might yield more authoritative results. Evidence from several sources and this project suggest that *P. virgatum* and *P. amarum* might be sexually compatible and might hybridize in the wild. Further studies are needed to verify this so that scientists and policymakers engaged in the ERA of transgenic switchgrass can take transgene flow between species into account.


Figure 1: Flowering periods of New England *Dichanthelium* species as compared with general flowering period of genus *Panicum* adapted from Barkworth et al, 2007. Most members of genus *Dichanthelium* exhibit two distinct flowering periods; the first characterized by open pollination (blue) and the second by Cleistogamous flowers (red).
Figure 2: Heights of five *Panicum* and three *Dichanthelium* species measured from the top of the soil to the tip of the longest leaf (n=5).
Figure 3: Heights of five *Panicum* and three *Dichanthelium* species measured from ligule to blade tip (n=5).
Figure 4: Blade widths of five *Panicum* and three *Dichanthelium* species measured at the midpoint.

(n=5).
Figure 5: Mean number of shoots per plant measured in five *Panicum* and three *Dichanthelium* species (n=5).
Figure 6: Mean number of panicles per plant in five *Panicum* and three *Dichanthelium* species (n=5).
Figure 7: Mean number of spikelets per inflorescence in five *Panicum* and three *Dichanthelium* species (n=5).
Figure 8: Estimated seed yield per plant for five *Panicum* and three *Dichanthelium* species calculated as the product of the number of spiklets and number of inflorescences (n=5).
Table 1: Panicles successfully crossed or tested for self compatibility. (*) Indicates that a cross from this set was assessed using 18 SSR molecular markers.

<table>
<thead>
<tr>
<th>Female Parent</th>
<th>Male Parent</th>
<th>Number of Bagged Panicles</th>
<th>Mature Caryopsis Produced By</th>
<th>Seeds/Flowers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. virgatum</em> 'Blackwell'</td>
<td><em>P. amarum</em></td>
<td>11</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td><em>P. virgatum</em> 'Alamo'</td>
<td><em>P. amarum</em></td>
<td>3</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td><em>P. virgatum</em> (North Carolina)</td>
<td><em>P. amarum</em></td>
<td>4</td>
<td>Yes</td>
<td>22/60*</td>
</tr>
<tr>
<td><em>P. amarum</em></td>
<td></td>
<td>6</td>
<td>No</td>
<td>2/125 ; 4/283</td>
</tr>
<tr>
<td><em>P. virgatum</em> 'Alamo'</td>
<td><em>P. dichotomiflorum</em></td>
<td>15</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td><em>P. virgatum</em> (North Carolina)</td>
<td><em>P. anceps</em></td>
<td>6</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td><em>D. meridianal</em></td>
<td></td>
<td>4</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td><em>D. commutatum</em></td>
<td></td>
<td>5</td>
<td>No</td>
<td>51/81</td>
</tr>
<tr>
<td><em>P. virgatum</em> 'Alamo'</td>
<td></td>
<td>3</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td><em>P. anceps</em></td>
<td></td>
<td>5</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td><em>P. virgatum</em> (North Carolina)</td>
<td></td>
<td>5</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 5

Distribution of Switchgrass (*Panicum virgatum* L.) Genotypes in Coastal Habitats and Inland Road Corridors
Abstract

Introduction: Corridors such as road verges can play an important role in plant distribution, dispersal, and patterns of genetic diversity. Switchgrass (Panicum virgatum) is an outcrossing grass with various ploidy levels and ecotypes across its North American range. In the northeast, it has become established in road verges, although it is not known if these plants are local genotypes or introduced through human activity. This study was designed to test two ideas: 1) that coastal and roadside switchgrass plants share one local genotype, and 2) a local genotype can be defined and is related to other East Coast genotypes.

Methods: Eighteen simple sequence repeat (SSR) molecular markers were used to infer genetic relationships between three groups of plants: 86 switchgrass plants collected from the Long Island Sound Coastal Lowland ecoregion, 37 plants collected from road corridors in the adjacent inland ecoregions, and individuals from 28 cultivars representing ecotype and ploidy groups from North America. DNA repeats were used in principal coordinates analysis and STRUCTURE to find similarities between collected plants and the cultivar genotypes.

Major results: Analysis of the SSR markers revealed that the majority of plants collected from the inland road verges (70%) grouped with Upland octoploid cultivars (Cave-in-Rock, Shelter), while the majority of plants collected in the Long Island Sound Coastal Lowland (88%) grouped with Lowland tetraploids. In additions, the DNA SSR marker from 61 coastal individuals did not match any cultivar comparators, but appeared most similar to Lowland tetraploid ecotypes from the East Coast between New York and Florida. The northeastern coastal plants in this study
appear to represent a distinct local genotype. Thus the northeastern coastal plants in this study appear to represent a local genotype designated Southern New England Lowland Tetraploids (SNELT).

Major conclusions: The majority of switchgrass plants collected from inland roads were Upland octoploids leading to a rejection of the hypothesis that native, coastal switchgrass plants had expanded their range along inland road verges. Most of the roadside plants were non-local genotypes (Upland octoploids). The simplest explanation for their dominance along roads is that they were introduced through human activity and were well adapted to road verges.

Abbreviations: Upland octoploid, U8x; Lowland tetraploid, L4x; Long Island Sound Coastal Lowland ecoregion, LISCL.
Introduction

Natural and anthropogenic corridors, such as coastal grasslands and road verges, are linear habitats that can affect species distribution plant population genetics (see book chapter by Meffe & Carroll, 1994). Road verges are among the most common habitat type in human-developed ecosystems, and they generally differ from the surrounding communities in water run-off, drainage, light availability, soils, and other characteristics (Christen & Matlack, 2009). Road verges are, however, affected by various types of disturbance (e.g. mowing, construction) and edge effects. Road verges can provide hospitable habitat for the spread of invasive exotic species (Pauchard & Alaback, 2004) or habitat reserves for native plant species (Karim & Mallik, 2008). Movement along these corridors can be through random diffusive processes or nonrandom dispersal events (Wilson et al., 2009). In particular the thoroughly interconnected nature of road networks encourages widespread dispersal (Jodoin et al., 2014; Lelong et al., 2007). Vehicles, animals, and wind can facilitate seed and pollen movement along road verge corridors. Wind-dispersed seed can be pulled along in eddies by trains and vehicle traffic and has been shown to play a role in seed dispersal (Christen & Matlack, 2009; Kalwij et al., 2008). The assumption that road verges serve as dispersal corridors is widely accepted, however, the appearance of a relationship between road verges and species distribution may be an artifact of observations limited to public roads and distribution data from a single point in time. Species distributions change over a long time scale, so conclusions about the dispersal process are difficult. The corridor function of road verges is of growing interest to ecologists, land managers, and risk analysts. While it is clear that road verge networks can facilitate dispersal of plant species there affects on the population genetics of those species is still unclear. There is no consensus about the effect of corridors on dispersal or population genetics of native, exotic, or invasive plant species. A study in Canada of the invasive, exotic genotype of Phragmites australis concluded
that the development of a highway network strongly contributed to its inland expansion (Lelong et al, 2007). Conversely, a study on weedy *Raphanus raphanistrum* population genetics in South Africa reported that road verges did not act as dispersal corridors (Barnaud et al, 2013). Overall, relatively few studies have examined the function of road verges with regard to changes in native plant distribution (Holderegger & Di Giulio, 2010), and fewer still have used molecular tools to distinguish local and exotic genotypes of the same species. These intraspecific distinctions may be difficult or impossible to distinguish morphologically, making the use of molecular tool vital. In addition, local and exotic populations may exhibit different traits making distinguishing between them functionally important (Saltonstall, 2002).

Switchgrass (*Panicum virgatum* L.) is a warm season, perennial grass native across much of North America. It is native to the East Coast and Gulf of Mexico, as well as tall grass prairies in the Midwest (Weaver & Fitzpatrick, 1934). In the Northeastern region, the presettlement distribution of switchgrass is believed to have been the narrow zone just upland of the coastal salt marsh (Niering and Warren 1980). However, two recent studies in the Northeastern region (Ahrens et al, 2011, Ahrens et al, in review) found the presence of switchgrass in road verge plant communities was positively correlated with urban areas (defined as at least 1000 persons mile$^{-2}$) and at distances up to 188 km from the coast. This has raised questions about the possibility of coastal plants dispersing inland along highway networks.

Determining the origin of ‘wild’ switchgrass plants is complicated due to a history of introduced genotypes and breeding programs that have provided seed for forage, wild life restoration and ornamentals. In recent decades, considerable attention has been given to switchgrass improvement to develop a low-input lignocellulosic biofuel crop using both traditional breeding techniques and genetic engineering (GE) (Parrish & Fike, 2005, Wright &
The application of genetic engineering has raised questions about the potential for gene flow between cultivated GE biofuel fields and other switchgrass populations with subsequent harm to valued environmental endpoints (e.g. communities, ecosystems) (NAS, 2002). Ecological risk assessments for GE switchgrass must specifically attend to unintended gene flow and its consequences because grasses can have characteristics associated with high levels of gene flow including: minimal domestication; shared habitat with sexually-compatible wild relatives; related taxa that are aggressive weeds or invasives, and flowers that are out-crossing and self-incompatible (Wipff, 2002). Switchgrass has not undergone intensive breeding with most cultivars separated from wild populations by only a few breeding cycles (Zalapa et al., 2011). North America is home to other Panicum species including agricultural weeds (e.g. *P. capillare* & *P. dichotomiflorum*) (Uva et al., 1997). Switchgrass is self incompatible and out-crossing (Martinez-Reyna & Vogel, 2002). Collectively, these factors highlight the potential for unintended gene flow (transgene movement) to cultivated, feral, or native populations.

Switchgrass is divided into two ecotypes; Lowland individuals are usually larger and tetraploid, while Upland individuals tend to be smaller and either tetraploid or octaploid (Martinez-Reyna and Vogel 2002; Porter 1966). Upland plants are associated with dry to moderate moisture habitats, while Lowland plants are common in both riparian and coastal flood plains (Ahrens et al., 2011; Porter, 1966). These ecotypes are thought to correspond broadly with the glacial refugia posited by McMillan (1959). Upland ecotypes can be further divided into a northern tetraploid lineage stretching from North Dakota to New York, and two octoploid lineages developing from the Great Plains and Gulf Coast. Lowland ecotypes separate into two lineages from the southern Great Plains and the eastern Gulf Coast (Zhang et al., 2011a). Recent
genomic analysis reaffirms many of these conclusions and identified the Northeastern region as home to Lowland tetraploid populations distinct from the southern Lowland tetraploid populations that have been studied (Lu et al., 2013). Unlike many previous studies of switchgrass population structure and genetic diversity which have focused on regional or continental scales, several recent examinations have focused on small regions and landscape level patterns of genetic diversity. Morris et al. (2011), using genomic tools, found little nuclear diversity among populations sampled from Indiana dune habitats, though significant plastome genetic variation was detected. Nageswara-Rao et al., (2012) used RAPD markers to compare natural switchgrass populations in Tennessee with cultivars ‘Alamo’ and ‘Kanlow’ and found that individual populations were readily distinguishable. Muteigi et al., (2013) assessed a number of cultivars against remnant populations from Illinois and Ohio. They found that populations grouped by state of origin and that ‘Cave-in-rock’ and ‘Shelter’ were most closely aligned with those from Illinois (Cortese et al., 2010; Jarne & Lagoda, 1996; Narasimhamoorthy et al., 2008; Zalapa et al., 2011; Zhang et al., 2011a).

The purpose of this study was two-fold: 1) determine if switchgrass plants observed in the Long Island Sound Coastal Lowland ecoregion were native genotypes from pre-settlement switchgrass populations, and 2) determine if plants observed in road verges were the result of range expansion from coastal populations or cultivars developed from non-local gene pools. Our hypothesis was that native, coastal switchgrass plants had dispersed from the coastal zone to inhabit the favorable corridors in road verges. To address these questions, individuals were collected and SSR molecular markers were used to infer their genetic identity and putative regional origin. This information will inform land managers and risk analysts as GE switchgrass is considered for wide spread commercial release and may also prove valuable to
conservationists and others wishing to protect and promote native species and genotypes. This study may be particularly useful as both the region (northeastern North America) and habitats (coastal and inland) on which it focused have received much less research attention in switchgrass than the prairie habitats of the plains.

Materials and Methods

The study site was located within the western boundary of (-73.500621), eastern boundary of (-71.468331), northern boundary of (42.111813), and southern boundary of (41.04363). Spatial information about collection sites was obtained using a Juno SB GPS unit (Trimble Navigation Limited, Westminster, CO, USA) and processed using ArcGIS 10.2 (ESRI, Redlands, CA). Spatial layers for ecoregions and roads were obtained from the United States Environmental Protection Agency (EPA) and the Connecticut Department of Energy and Environmental Protection respectively (“Connecticut Routes,” 2003; Griffith et al., 2009).

Switchgrass collection was informed by two previous studies on its habitats and distribution in two Level III Ecoregions: the Northeast Coastal Zone, and Northeastern Highland Zone (Figure 1) (Ahrens et al., 2011, Ahrens et al, in review). The Level IV sub-ecoregions in the study site were: 59g Long Island Sound Coastal Lowland (LISCL), 59a Connecticut Valley, 59b Lower Worcester Plateau/Eastern Connecticut Upland, 59c Southern New England Coastal Plains and Hills, 58d Lower Berkshire Hills, and 58e Berkshire Transition. In the LISCL coastal subecoregion, 87 plants were collected at 35 locations in three habitat types previously shown to support switchgrass: semi-natural, human impacted, and road verges (Ahrens et al., 2011). In some coastal sites, three individuals were collected when each plant was at least 2 m apart to minimize the possibility of sampling more than once from a single genet. Preliminary botanical
surveys were conducted along 14 inland roads roughly perpendicular to the Long Island Sound coastline and containing four or more switchgrass populations. From this group, five inland roads were selected for collecting individual switchgrass plants at least 1.6 km apart and within 3 m from the edge of the road. One plant was collected from each site for a total of 37 individuals.

Eighteen SSR markers were used to analyze the DNA from 124 collected switchgrass plants and 59 individuals representing 25 cultivars (Table 1). These results were aligned and combined with electropherograms from a previous study that included 182 individuals from 18 cultivars (Table 1). This combined dataset served as the basis for analysis of the collected individuals. Seed for 20 cultivars was acquired from the USDA National Genetic Resources Program (www.ars-grin.gov), Ernst Seeds (Meadville, PA, USA), or Sharpe Brothers Seed Company (Clinton, MO, USA). Five ornamental cultivars were obtained as vegetative propagules from Broken Arrow Nursery (Hamden, CT, USA). All collected plants and cultivars were maintained in the University of Connecticut greenhouses.

DNA extraction involved processing 100 mg frozen leaf tissue with a TissueLyser II (Qiagen, Valencia, CA, USA) followed by extraction using the DNeasy Plant Mini kits (Qiagen, Valencia, CA, USA). Eighteen previously-published primer pairs (Zalapa et al, 2011) were synthesized by the University of Wisconsin Biotechnology Center and used in PCR according to published protocols (Zalapa et al., 2011). The SSR primer pairs included SWW: 112, 151, 185, 432, 438, 439, 593, 651, 664, 686, 2309, 2312, 2341, 2385, 2394, 2415, 2416, 2431.

DNA fragment analysis was performed by Cornell University, Institute of Biotechnology using an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). In addition to the analysis of collected switchgrass plants and 21 cultivars, electropherograms for 182 individuals were obtained from Michael Casler (University of Wisconsin) and analyzed.
Genotypes were derived from scoring of alleles using GeneMarker v 1.95 (Softgenetics, State College, PA, USA). Because switchgrass is a polyploidy species, SSR data was transformed from binary data to fragment sizes and analyzed in relation the corresponding primer pair using polysat version 1.3-2 in R (Clark & Jasieniuk, 2011). Principle coordinate analysis (PCA) was conducted in polysat using Bruvo distances (Bruvo et al., 2004). Polymorphic information content (PIC) was calculated using PICcalc (Nagy et al., 2012). Analysis of molecular variance (AMOVA) was performed in GenAlEx to determine the partitioning of diversity within and among ecoregions, ecotype/ploidy groups, and cultivars (Peakall & Smouse, 2006). Alleles were analyzed using Bayesian inference (STRUCTURE 2.3.4) in which Bayesian clustering algorithms were used to iteratively group individuals by putative genotype (Pritchard et al., 2000). STRUCTURE was run using the ‘admixture model’ and 25,000 Markov chain Monte Carlo iterations with 10,000 burn-in iterations and 10 replicates per run. Optimal K values for STRUCTURE output were calculated using Evanno’s delta K implemented in Structure Harvester (Earl & vonHoldt, 2011).

**Results**

**Genetic diversity**

The 18 primer pairs generated 156 distinct amplified fragments (mean 30.4, range=8-48 fragments per individual) with a mean 8.7 fragments per primer pair (range=4-23) (Table 2). The PIC value across all primer pairs (loci) ranged from 0.79-0.96 with an mean of 0.91; this mean was higher than previously reported for a larger set of 55 primer pairs (mean=0.66, Zalapa et al, 2011) or 19 primer pairs (mean PIC of 0.71, Zhang et al, 2011a). This suggested that the 18 loci used in this study were slightly more informative than the loci used in previous switchgrass studies. The number of different fragments and private alleles for each ecotype/ploidy group are
shown in Table 2. The largest number of private alleles was found in the U8x and L4x cultivar groups; collected switchgrass plants had 3-4 private alleles, while two U4x cultivars (‘Dakotah’, ‘Summer’) had no private alleles. While the LISCL coastal switchgrass plants are believed to be L4x (see below), it is reasonable to believe that the larger number of private alleles among the L4x ecotype/ploidy group could be due to their origin from a broader geographic region including the East Coast and the Gulf of Mexico region.

The heterozygosity and genetic diversity of switchgrass is such that a large sample size and many different lineages is necessary to determine the identity of individuals with unknown parentage or geographic origin. STRUCTURE analysis (k=9) of the comprehensive dataset (368 individuals) confirmed the genetic assignments observed in previous studies (Zalapa et al., 2011; Zhang et al., 2011a, 2011b) (Figure 2). The 128 collected individuals were grouped into five lineages (Figure 2): 28 individuals were U8x-B as represented by ‘Cave-in-Rock’ and ‘Shelter’; 19 individuals were L4x-C as represented by ‘Miami’; two individuals were L4x-D as represented by ‘Alamo’ and ‘Timber’; one individual was U8x-B as represented by ‘Pathfinder’, ‘Shawnee’, ‘Blackwell’, ‘Carthage’, ‘Trailblazer’. Nine of the collected individuals could not be assigned to a single lineage. Of greatest interest were the 61 individuals that did not group with any of the cultivar comparators. We have named this group Southern New England Lowland Tetraploid (SNELT). None of the SSR markers yield unique alleles associated with the SNELT group.

Polyploid data (Polysat)

One of the noteworthy attributes of the analysis used in this study was the retention of independent loci and polyploid allele numbers. This is contrast to other resent switchgrass studies in which molecular markers have been used but the complexities of analyzing polyploid
data have been side stepped by treating the allelic data as if it were haploid with each individual allele credited with its own locus.

Switchgrass from the Long Island Sound Coastal Lowland Ecoregion

Eighty-six switchgrass plants were collected in the LISCL subecoregion in road verges, disturbed coastal areas, and semi-natural habitats and 69% (59 plants) were assigned to the SNELT group. Flow cytometry was conducted on 20 SNELT individuals (based on SSR markers) and all appeared to be tetraploids when compared to L4x or U8x cultivars (T. Williams, data not shown). PCA analysis was used to compare the SNELT individuals to seven L4x cultivars (‘Miami’, ‘Wabasso’, and ‘Stuart’) that represent the ‘Florida clade’ (Zhang et al, 2011a, Zalapa et al, 2011). The SNELT plants remained distinct from four other L4x cultivars. However, STRUCTURE analysis of an exclusively L4x subset of the data (Figure 4) showed a more complex picture with the majority of the individuals collected from the LISCL (40 plants) distinct from the ‘Florida clade’ cultivars and 4 individuals grouped with the cultivar ‘Miami’. This provides evidence that a distinct Lowland tetraploid regional lineage was identified in the LISCL. However, some genetic diversity was observed with one individual having affiliation with the U4x-D group and one unknown affiliation.

Switchgrass from inland road verges

Thirty-seven switchgrass plants from inland road verges were analyzed and the majority of these individuals grouped with U8x cultivars (Figure 5). However, eight individuals grouped with the unique SNELT group (L4x). Thus, the SNELT genotype was detected at on some inland
road verges. In the next PCA analysis, the inland plants were compared with U8x and L4x cultivars (Figure 6). Eight individuals again grouped with the L4x cultivars and one plant was indeterminate in its assignment. Twenty-five plants grouped with two U8x cultivars ‘Cave-in-rock’ (CIR) and ‘Shelter’. As in other studies, we were unable to distinguish between these two cultivars because both represent a genotype derived from the Ohio River Valley and the Central Appalachian Mountain Region, and ‘Shelter’ was derived from ‘Cave-in-Rock’ (Vogel, 1996; Mutegi et al., 2013; Zalapa et al., 2011). Three other plants grouped with all other U8x cultivars. STRUCTURE analysis gave a similar result (Figure 7) showing that inland road verges contained a mixture of U8x individuals (CIR, Shelter) and L4x plants similar to the Florida clade and putatively native within the study site.

AMOVA was conducted on two groups of plants: all collected plants, or individuals representing 18 cultivars. Results indicated that the majority of genetic diversity was detected within rather than between (among) ecoregions, cultivars, or ecotype/ploidy groups (Table 4).

**Spatial distribution of switchgrass genetic groups**

The botanical survey confirmed previous observations (Ahrens et al, 2011, Ahrens et al, in review) regarding the presence of switchgrass populations in two coastal habitat types (disturbed, semi-natural) and road verges in Ecoregion Level III Zone 59 (Northeastern Coastal zone, NC) and Zone 58 (Northeastern Highlands zone, NH) (Figure 1). When the ecotype/ploidy levels identified for the collected plants were mapped to the study site, a general pattern was observed. The LISCL region had a large percentage of SNELTs (69% of individuals) while the inland region had a large percentage of plant grouped with U8x cultivars (70% of individuals) (Figure 8). The strongest discernible distribution pattern was in a North-South orientation with
U8x plants towards the north (inland) and SNELTs towards the coastal zone. However, mixtures were observed in both regions with two U8x individuals only 7 and 13 km from the Long Island Sound, and two SNELT individuals detected at 40 and 44 km from the coast. Plants without a clear membership in any genetic group were found in both regions; three were on inland road verges and six were in the coastal zone. Further research would be required to determine if these individuals were hybrids.

**Ornamental cultivars**

Switchgrass cultivars have been developed for their ornamental traits, but they have never been included in genetic studies. Four ornamental cultivars were included in this study (Table 1) because of the presence of urban landscapes and the possibility that garden plants could escape cultivation. SSR marker results showed that ‘Dallas Blue’ and ‘Northwind’ had primary membership probability with the L4x cultivar ‘Miami’. ‘Haron Salstius’ grouped with the U8x cultivars ‘Sunburst’ and ‘Forestburg’. Cultivars ‘Ruby Ribbons’ and ‘Shenandoah’ showed mixed genetic assignment. Thus, three of the five ornamental cultivars were closely aligned with well-defined switchgrass ecotype/ploidy groups.

**DISCUSSION**

**Road Verge Corridors**

The hypothesis for this study was that road verges acted as corridors for dispersal of native *Panicum virgatum* (switchgrass) populations from their pre-settlement habitat type in the Long Island Sound Coastal Lowland ecoregion. The SSR molecular markers indicated that 70% of the inland switchgrass individuals collected from road verges were most similar to the U8x-A
cultivars ‘Cave-in-Rock’ and ‘Shelter’ (Fig. 8, Table 3). The lineage of these cultivars is associated with the Ohio River Valley and the Central Appalachian Mountain Region (Zhang et al., 2011a). Only 22% of road verge plants could be grouped with the coastal genotype. Thus, the evidence did not strongly support the hypothesis that coastal switchgrass lineages have expanded their distribution along road corridors. Instead, a more complex picture arose regarding the genotypes of the current populations. For example, plants collected from the I-95 road verge were predominantly from the local coastal genotype consistent with individuals collected from the same LISCL ecoregion (Figure 8). The presence of U8x populations and local coastal genotypes in the road verge network could prove important for managing gene flow future and the conservation of genetic resources.

**Coastal Populations**

In the Long Island Sound Coastal Lowland (LISCL) switchgrass is a component of the plant community occupying the narrow zone upland and adjacent to the coastal salt marsh (Niering and Warren, 1980). Since much of this coastal habitat has been altered by human activity, one of the goals of this study was to determine the lineage of switchgrass populations relative to genotypes from other parts of North America. Molecular studies have characterized switchgrass population genetics (e.g. lineage, ploidy, and ecotype) across its range in North America although the geographic origin of samples and molecular methods differed (Cortese et al., 2010; Lu et al., 2013; Zhang et al., 2011a). Cortese et al. (2010) collected switchgrass in New York and these plants grouped most closely with ‘High Tide’ a L4x cultivar. Lu et al. (2013) collected switchgrass from New York including Long Island and suggested that these individuals belonged to a “Lowland 4x Northeast” group. However, this study could not make a connection
with the L4x ‘Florida Clade’ identified by Zhang et al (2011a) because it did not include accessions from the Southeastern Atlantic or Gulf Coast (Florida, South Carolina, North Carolina). The study by Zhang et al. (2011a) included samples for the Southeastern region; however they did not include accessions north of New York City and New Jersey. Taken together, the results of these studies suggest that the ‘Northeastern lineage’ (represented by samples from New York State including Long Island) is L4x and derived from refugia in the Southeastern Atlantic or Gulf Coast region during the last ice age.

In our study the majority of the switchgrass plants (88%) collected from the coastal LISCL subecoregion in this study represented a distinct genotype that we have designated the Southern New England Lowland Tetraploid (SNELT). These plants were distinct from the four L4x-D cultivars used as comparators (Table 1). SNELT plants showed the closest genetic assignment to L4x cultivars of the Florida Clade (L4x-A and L4x-C) (Zhang et al, 2011a). Thus, the SNELT plants had the same lineage as previously reported for coastal switchgrass plants collected farther south (e.g. New York, New Jersey) supporting the theory that L4x populations from the Southeast moved northward after the last ice age (Lu et al., 2013; Zhang et al., 2011a). Previous studies in the southeast and midwest US have also detected distinguishable local genotypes in fragmented populations (Mutegi et al., 2013; Nageswara-Rao et al., 2012; Morris et al., 2011).

There are several lines of evidence that suggest the L4x SNELT plants collected from the coast represent the local genotype. First, the SNELT plants were collected from the periodically flooded soil conditions favored by the Lowland ecotype (Barney et al., 2009; Porter, 1966). Second, SNELT individuals were genetically distinct from the most common L4x cultivars sold by seed companies. Comparators in this study included older L4x cultivars ‘Alamo’ (1978) and
‘Kanlow’ (1963) and newer L4x cultivars ‘Timber’ (2009), ‘BoMaster’ (2006), and ‘Performer’ (2006) (Casler, 2012). Among the SNE LT individuals, 12 plants showed a close genetic affiliation with the L4x-A Florida Clade cultivar ‘Miami’ while remaining distinct from the representatives of the L4x-C Florida Clade ‘Wabasso’ and ‘Stewart’. These three cultivars originated around 1996, but they are not common seed mixes today. Thus, it is unlikely that the SNE LT individuals were the result of human introduction in the LISCL subecoregion. The detection of the local SNE LT genotype is encouraging and future projects should preserve genetic this genetic resource for future breeding programs. Coastal restoration projects in the LISCL ecoregion should use cultivars from the Florida clade or seek cultivars from SNE LT populations (Gustafson et al., 2004; Moncada et al., 2007). Furthermore, this study suggests that restoration projects in the LISCL ecoregion seeking to maintain the local genotype should seek cultivars from the Florida Clade or specific coastal sites now known to contain SNE LT populations.

**Spatial Distribution of Genotypes**

This study examined switchgrass genetics across two ecoregions and two habitat types road verges and coastal sites. There are several potential explanations for the observed pattern genotypic distribution. The simplest explanation is that ‘Cave-in-Rock’ or ‘Shelter’ were planted as part of road verge seed mixes. Switchgrass has long been recommended as a roadside planting due to its ecological functions and adaptability (Brown et al., 2011). The second explanation could be that switchgrass was cultivated elsewhere and seed spread to the roadsides. Both scenarios can explain the frequency of none local U8x genotypes. Subsequently the adaptability of the U8x cultivars benefited from increased cold hardiness and decreased mortality in severe
winter temperatures (Casler et al., 2002; Casler et al., 2004). U8x plants could also have been favored by well drained soils in road verge habitats (Barney et al., 2009). Temperature in late summer plays a large role in switchgrass flowering and reproduction, it is possible that inland temperatures are more favorable for U8x plants than other genotypes (Balasko & Smith, 1971). A recent modeling study suggested that minimum winter temperature was an important explanatory variable in switchgrass distribution in northeastern North America (Ahrens et al., In Review).

A third potential explanation for the observed genotypic distribution pattern is that we have discovered a natural transition zone between L4x and U8x genotypes. A map drawn by Casler (2012) designated as transition zone between Upland and Lowland ecotypes that encompasses our study site. Our study site falls near both the transition zone between the native ranges of switchgrass ecotypes and the border between regional gene pools (Casler, 2012). However, there is no documentation suggesting that switchgrass grew inland prior to European settlement, and extensive road corridors did not exist until recent times.

Conclusions: Climate change, coexistence, and native genetic resources

If temperature is the main influence deterring SNELT plants from establishing road verge populations in inland ecoregions, then climate change may allow for a greater degree of inland establishment. In addition to considering potential future changes in distribution, policy makers must also consider reproductive compatibility and the distribution of receiving populations. The producers and regulators of genetically engineered plants generally site field trials and large scale agricultural production of GE plants a significant distance from reproductively compatible populations; the presence of which might facilitate transgene escape. The existence of
switchgrass populations across the region may make deployment of GE plants inadvisable where concerns over transgene escape persist. In particular, our findings suggest that both tetraploid and octoploid switchgrass populations are present and therefore introduced plants of either ploidy will have receptive populations with which to breed. However, if the main objective of policy makers is the preservation of local genotypes, the introduction of octoploid GE switchgrass cultivars would present a relatively low risk of gene flow to native L4x SNELT populations.

Acknowledgements

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Literature Cited


Figure 1: Map of the study site showing the locations for switchgrass collection (open circles). Black lines represent borders of Connecticut and Rhode Island and six roads (63, 8, 9, 95, 84, 2). Grey lines represent the borders of Level IV subecoregions. The shaded area represents Level IV subecoregion 59g Long Island Sound Coastal Lowland.
Figure 2: STRUCTURE analysis based on SSR markers of 21 switchgrass cultivars and collected plants sampled from 85 sites (n=368). Individual samples are represented by a vertical line corresponding to the membership fractions in each of the K populations and sorted according to q-value (K =9). Marked groups (*) include collected plants.
Figure 3: Principle coordinate analysis of individuals assigned as SNELT with seven L4x cultivars using Brovo distances. Analysis was conducted in R package polysat using Brovo distances. The circle indicates the clustering of coastal SNELT plants compared to the L4x cultivars.
Figure 4: STRUCTURE analysis of coastal individuals and 7 L4X cultivars, individual plants sampled from each site in the Coastal Lowland ecoregion (n=46) in the Northeastern US. Individual samples are represented by a vertical line corresponding to the membership fractions in each of the K populations (K=6).
Figure 5: Principle coordinate analysis showing assignment of inland collected individuals to two ecotype/ploidy groups. Analysis includes individuals from the inland subecoregions and eighteen cultivars. Colors indicate ecotype/ploidy group: U4X (Red), U8X (Purple), L4X (Green), Inland (Black).
Figure 6: Principle coordinate analysis showing similarity of some individuals with ‘Cave-in-rock’ and ‘Shelter’ U8X. Analysis includes Inland individuals and sixteen cultivars (U4X cultivars are excluded). Analysis was conducted in R package polysat using Brovo distances. Colors indicate group: ‘Cave-in-rock’ and ‘Shelter’ (Red), Remaining U8X (Blue), L4X (Green), Inland (Black).
Figure 7: STRUCTURE analysis of coastal individuals and 16 cultivars (U4X cultivars are excluded), individual plants sampled from each inland roadside site (n=37) in the Northeastern US. Populations are numbered as: ‘Cave-In-Rock’ (1), ‘Pathfinder’ (2), ‘Shelter’ (3), ‘Shawnee’ (4), ‘Sunburst’ (5), ‘Forestburg’ (6), ‘Blackwell’ (7), ‘Carthage’ (8), ‘Trailblazer’ (9), ‘Kanlow’ (10), ‘Alamo’ (11), ‘SG5’ (12), ‘Timber’ (13), ‘Wabasso’ (14), ‘Miami’ (15), ‘Stuart’ (16), Inland (17). Individual samples are represented by a vertical line corresponding to the membership fractions in each of the K populations (K=6).
Figure 8: Distribution of switchgrass ecotype/ploidy groups. Map of the study site showing the locations for switchgrass collection (colored circles). Black lines represent borders of Connecticut and Rhode Island and six roads used in the botanical survey. Grey lines represent the borders of Level IV subecoregions. The shaded area represents Level IV subecoregion 59g Long Island Sound Coastal Lowland.
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<tr>
<td>Coastal</td>
<td>Connecticut /Rhode Island</td>
<td>4x</td>
<td>-</td>
<td>87</td>
<td></td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1: Cultivars and collected plants used in this study (Origin, Ecotype, and ploidy are shown based on previous reports). Including US state of origin, ecotype, ploidy, collected and/or tissue extracted samples (TE), and reanalyzed electropherograms (RE) (Zalapa et al., 2011). Lineage based on the majority genotype assigned population in (Zhang et al., 2011a). USDA National Genetic Resources Program (GRIN); Ernst Seeds (Ernst); Sharpe Brothers Seed Company (Sharpe); and Broken Arrow Nursery (BAN).
Table 2: Genetic diversity characteristics of switchgrass in this study based on 18 SSR loci.

<table>
<thead>
<tr>
<th>Population</th>
<th>All individual</th>
<th>U4x</th>
<th>U8x</th>
<th>L4x</th>
<th>Coastal</th>
<th>Inland</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Alleles</td>
<td>156</td>
<td>87</td>
<td>129</td>
<td>115</td>
<td>104</td>
<td>112</td>
</tr>
<tr>
<td>Number of Alleles Freq. ≥ 5%</td>
<td>97</td>
<td>73</td>
<td>95</td>
<td>78</td>
<td>66</td>
<td>90</td>
</tr>
<tr>
<td>Number of Private Alleles</td>
<td>-</td>
<td>0</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2: Genetic diversity characteristics of switchgrass in this study based on 18 SSR loci.
<table>
<thead>
<tr>
<th>Region</th>
<th>L4X</th>
<th>U8X</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long Island Coastal Lowland</td>
<td>88%</td>
<td>8%</td>
<td>4%</td>
</tr>
<tr>
<td>Inland Ecoregions</td>
<td>22%</td>
<td>70%</td>
<td>8%</td>
</tr>
</tbody>
</table>

Table 3: Percentage of collected individuals from coastal and inland regions by ecotype/ploidy groups. Unknown group includes individuals without a clear genetic assignment.
<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>%</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Collected Individuals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between Inland and Coastal</td>
<td>1</td>
<td>204.88</td>
<td>204.88</td>
<td>0.233147</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Within Inland or Coastal</td>
<td>123</td>
<td>1496.555</td>
<td>12.16711</td>
<td>0.766853</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total</td>
<td>124</td>
<td>1701.435</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>(2) Cultivars</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among Ecotype/Ploidy</td>
<td>2</td>
<td>741.7149</td>
<td>370.8575</td>
<td>0.231414</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Among Cultivars</td>
<td>15</td>
<td>769.6335</td>
<td>51.3089</td>
<td>0.171198</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Within Cultivars</td>
<td>213</td>
<td>2434.237</td>
<td>11.42834</td>
<td>0.597388</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total</td>
<td>230</td>
<td>3945.585</td>
<td></td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Analysis of molecular variance (AMOVA): 1) within and among 59g (LISC) and all other Level IV subecoregions, and 2) within and among ecotype/ploidy groups (3) and as represented by cultivars.
Chapter 6

Concluding Thoughts

The research conducted for this dissertation focused on the distribution, genetic diversity, and pollen-mediated gene flow of switchgrass (*Panicum virgatum* L.) in the northeastern U.S. (Ecoregion Level III: Northeastern Coastal Zone, Northeastern Highlands). The studies were intended to inform ecological risk assessment (ERA) for the future cultivation of genetically engineered (GE) switchgrass. The results will be useful for decision-making by stakeholders working with both conventional and GE switchgrass including government regulators, plant breeders, land managers, seed producers, biotechnology companies, and farmers. These projects have also increased basic knowledge about the biology and ecology of switchgrass.

**ERA for GE Switchgrass is different than for GE Corn**

Although switchgrass has been in cultivation for many years, advances in its genetic engineering and adoption as a biofuel feedstock have outpaced the scientific understanding of its ecological functions, habitat distribution, pollen biology, species interactions, and species distribution. This situation places regulatory agencies, stakeholders and the public in an awkward situation. In the past, GE crops (e.g. corn, soybeans, cotton) approved by USDA have been plant species with a high degree of familiarity, extensive scientific information, and a long history of genetic manipulation and domestication (Ellstrand, 2003). For example, ERA for corn is relatively simple because it is an annual crop, highly domesticated, and dependent on human cultivation. Corn gene flow in North America involves pollen movement between cultivated corn fields, but there are no sexually-compatible native species or weedy relatives. While a small number of volunteer corn plants can emerge the second year, the plant does not form feral
populations in roadsides or natural areas. In short, corn is an annual that is highly dependent on direct human cultivation.

In contrast, switchgrass grows in various settings as a native plant, a cultivated plant, or a weedy plant in disturbed sites. Results of a botanical survey (Chapter 2) showed that switchgrass can be found in all three contexts in the northeastern US. Switchgrass responds well to various types of disturbance in ruderal, cultivated, and natural coastal habitats. It has little or no dependence on human management due to its adaptability, perennial life cycle, abundant seed production, and other features. Furthermore, the reproductive biology of switchgrass suggests a high probability for gene flow between cultivated switchgrass and other populations. Switchgrass produces abundant pollen and is an obligate outcrossing species. A Lagrangian model of switchgrass pollen dispersal suggested that normal summer wind conditions could carry viable pollen as much as 6.5 km (Chapter 3). The combination of wind-blown pollen and numerous receiving populations (natural, feral, cultivated) suggested that pollen-mediated gene flow could occur in the study region. Whether gene flow would cause negative ecological impacts would depend on many factors including the novel genes and traits spreading from the biofuels fields.

The botanical survey showed that switchgrass has a number of local congeneric species that have the potential for pollen-mediated gene flow. In particular, there could be problems if switchgrass formed hybrids with some Panicum species (P. miliaceum, P. dichotomiflorum, and P. capillare) that are already agricultural weeds. However, research in this area did not support the concept of interspecific hybridization.

**Protecting Local Switchgrass Genotypes**
This research was able to provide strong evidence for a local Lowland tetraploid (L4x) genotype in the fragmented coastal landscape of the Long Island Sound Coastal Lowland Level IV ecoregion. The presence of this local genotype should be of particular interest to risk analysts and conservationists interested in preserving native plant communities. The introduction of engineered or non-local genotypes for biofuels production could allow gene flow into the coastal switchgrass plants. It should be noted that the local genotype persists despite many years of coastal development and disturbance. In fact, the botanical survey and the collection of samples for DNA analysis were challenging because of due to the predominance of private lands along the coast and the high degree of development. In some ways, the fragmented coastal habitat in the northeastern US presents similar challenges to the remnant prairie habitat in the Midwest for the conservation of switchgrass germplasm.

The natural coastal habitat for switchgrass is adjacent to another coastal native \textit{Phragmites australis} (Common Reed). The botanical survey found both species (see Chapter 2) in the study region. It is worth exploring the story of \textit{Phragmites} as a cautionary tale for the effects of switchgrass gene flow. Modern molecular studies on \textit{Phragmites australis} in the US demonstrated that almost all of it is the result of hybridization with an exotic genotype (Saltonstall, 2002). The behavior of this hybrid \textit{Phragmites} is far more aggressive than its native predecessor, and gene flow has contributed to its development as an invasive species. This is relevant to remanant, coastal genotypes of switchgrass in several ways. First, the presence of hybrid \textit{Phragmites} and other invasive plants could pose a threat to the relatively small populations of native switchgrass in coastal Connecticut and Rhode Island. Second, the \textit{Phragmites australis} story is illustrative of the potential consequences of gene flow hybridization. Concerns about gene flow producing weedy switchgrass genotypes are often
dismissed by skeptics on the basis that it is a native plant and, to their way of thinking, ineligible for consideration as a potential future invasive. However, gene flow and the introduction of traits that increase fitness (e.g. salt tolerance, drought tolerance, or increased nitrogen use efficiency) to local switchgrass populations could lead to unpredictable future consequences.

The introduction of very large switchgrass biofuel plantations could, in theory, produce genetic swamping in coastal populations. Genetic swamping occurs when a large population contributes outsized pollen clouds and gene flow that, over time, alter the genetics in the smaller receiving population. Coastal switchgrass may be particularly susceptible to this because it is an obligate outcrossing species and local populations tend to be small (C. Auer and G. Ecker, personal observation). GE switchgrass could incorporate traits, such as reduced lignin, that facilitate cellulosic ethanol production but reduce overall plant structural integrity and plant fitness. Genetic swamping of small coastal populations could be problematic because these plants experience higher levels of mechanical stress (e.g. wind) than inland populations in field cultivation. Over time native populations could be reduced or become extinct due to genetic swamping.

**Coastal Switchgrass is often Overlooked**

Most literature refers to switchgrass as a major constituent of the tall grass prairie. While this is certainly true, it overlooks the large numbers of native populations that grow along the eastern seaboard of North America from the Gulf of Mexico to the coastal habitats of Canada. While distribution maps generally recognize the coastal populations, the major molecular genetics and phylogenetic studies have not included coastal samples north of New York, Long Island, or New Jersey. The prevailing emphasis on prairie switchgrass may be due to the predominance of research groups in the Midwest and Western states. This is unfortunate
because some molecular studies consider the Eastern Gulf Coast as the center of genetic diversity for the species (Zhang et al., 2011a) and the coastal populations clearly offer unique genetic resources (see Chapter 5).

Future Directions

While this research has made significant progress in elucidating the biology and dispersal of switchgrass pollen, a great deal of work still needs to be done in this area. For example, there are no good estimates of pollen source strength for switchgrass fields. This information is critical to estimating the probability of long distance, pollen-mediated gene flow events.

In recent years, a number of studies have sought to understand the genetic diversity of switchgrass across its entire range. The species has, however, received considerably less attention at the fine spatial scale. For instance, to the author’s knowledge, this dissertation contains the only study investigating the genetics of coastal populations at a regional scale. Future studies focusing on specific landscape types and populations could fill current gaps in knowledge and prove valuable to policy makers.

This and other studies suggest that switchgrass is sexually compatible with at *P. amarum* (bitter beach grass). The degree of compatibility between these two species should be investigated further as it could potentially play a significant role in unintended transgene flow in the future. In some areas, *P. amarum* is a ‘state listed’ species with an important role in the protection of coastal dunes. Furthermore, GE switchgrass could be adopted as a biofuel crop in other countries. In this case, it will be necessary to investigate switchgrass’s sexual compatibility with *Panicum* in other parts of the world. This may prove a daunting task as *Panicum* is a large genus. Overall, more scientific information on switchgrass and its relatives
will be needed in order to provide a strong basis for ecological risk assessments, co-existence strategies, protection of genetic resources, monitoring programs, and many different land management decisions.
Literature Cited


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