

Population Genetics and Seed Set in Feral, Ornamental *Miscanthus sacchariflorus*

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Ornamental grasses may become invasive weeds depending on their ability to naturalize and outcompete other species. *Miscanthus sacchariflorus* (Maxim) Franch. (Amur silvergrass) is a tall, self-incompatible, nonnative grass that has become naturalized in eastern North America, forming monospecific stands and raising concerns about its potential invasiveness. To understand the extent of clonal and sexual reproduction in feral populations, we examined their population genetic structure, seed production, and ploidy. We surveyed 21 populations in Iowa and Minnesota using eight polymorphic microsatellite markers. Only 34 multilocus genotypes (MLGs) were detected from a total of 390 samples. All of the study populations had more than one MLG, thereby allowing cross-pollination with near neighbors, but most were dominated by one or a few MLGs. Low genetic divergence suggests that all populations may have originated from similar cultivars. Cluster analysis showed that the six populations from Minnesota were extremely similar to each other, whereas the 15 populations from Iowa were somewhat more diverse. Seed production was quantified for 20 populations and ploidy for 11 populations. Average seed production was very low (< 0.30 seeds per panicle), although most populations did produce seeds. Because the populations were diploid (2x), they also may have the potential to hybridize with ornamental varieties of *Miscanthus sinensis* (Chinese silvergrass; eulaliagrass), a diploid close relative. Clonal growth, self-incompatibility, and spatial isolation of compatible clones may contribute to pollen-limited seed set in these populations. Low seed set may affect the rate of spread of feral *M. sacchariflorus*, which appears to disperse vegetatively as well as by seed. Although this species is not widely viewed as invasive, it is worth monitoring as a species that may become more widespread in the future.

Nomenclature: Amur silvergrass, silver banner grass, *Miscanthus sacchariflorus* (Maxim.) Franch.; Chinese silvergrass, *Miscanthus sinensis* Anders.

Key words: Clonal, genetic structure, low seed production, microsatellite markers, naturalized.

Many ornamental plant species have escaped cultivation and are now invasive in the United States and elsewhere (Reichard and White 2001). Invasive species can displace natives, alter community structure, change ecosystem processes, cause damage as agricultural weeds, and cost billions of dollars in control and eradication efforts (e.g., D'Antonio and Vitousek 1992; Li et al. 2004). Intention-

ally introduced plants typically do not become naturalized unless they can survive without cultivation and are planted repeatedly at many locations, creating strong propagule pressure on local habitats (Lockwood et al. 2005; Mack and Erneberg 2002; Minton and Mack 2010). It is also common for future invasive species to go through a lag time of years or decades after initial naturalization before becoming more abundant (Sakai et al. 2001). Possible reasons for such lag times include factors such as geometric population growth, changing environmental conditions, evolutionary adaptation, hybridization with related taxa, or a combination of these factors (Sakai et al. 2001). Thus, it is often unclear whether naturalized species that are not invasive will remain so. Knowledge about ecological factors and processes that drive the establishment and spread of naturalized species, and especially the transition to invasiveness, can aid in designing effective control and management strategies.

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Management Implications

Ornamental perennial grasses may have the potential to become invasive in areas where they can easily naturalize and disperse. In the nonnative genus *Miscanthus*, unwanted establishment of feral populations of ornamental *M. sinensis* (Chinese silvergrass) already has occurred in parklands and other areas, primarily in the eastern and southeastern United States. Its close relative, *M. sacchariflorus* (Amur silvergrass), also establishes feral populations, typically in more northern regions, but these stands are not as widespread as *M. sinensis*, nor do they produce abundant seeds. Low seed production could be a factor that limits population growth rates in feral *M. sacchariflorus*. The co-occurrence of genetically distinct individuals is a requirement for cross-pollination and seed set in this clonally reproducing, self-incompatible species. If several cross-compatible individuals occur in close proximity, this might lead to more abundant seed production and subsequent dispersal to other sites. In the current study, we found that feral populations of *M. sacchariflorus* in Iowa and Minnesota are genetically similar and highly clonal, but more than one genetic individual was present at each of our study sites. At present, we do not know whether *M. sacchariflorus* is in a temporary “lag phase” that precedes greater invasiveness. In any case, this tall, vigorously clonal perennial is able to establish extensive monospecific stands that crowd out other species, which is why managers of natural areas typically try to eradicate newly established populations. Ornamental plantings of *M. sacchariflorus* continue to be popular in northern areas of the United States and in southern Canada; this species is more cold tolerant than *M. sinensis*. The silvery inflorescences of *M. sacchariflorus* are attractive for landscaping, and the plant is easy to cultivate, but it has the potential to become a nuisance. Therefore, it is prudent to monitor the surrounding area for feral volunteers, which are much easier to eradicate at an early stage compared with after they have propagated clonally to form large stands along ditches, roadsides, wet meadows, and other areas.

Here we focus on the population genetics, clonal genetic diversity, and seed production of a naturalized, clonal ornamental grass that appears to be expanding its range in the United States and elsewhere, despite having very low seed set (Bonin et al. 2014; Schnitzler and Essl 2015). *Miscanthus sacchariflorus* (Maxim.) Franch., known as Amur silvergrass or silver banner grass, is a perennial C_4 grass that was probably introduced to North America from Asia in the 1930s (Bonin et al. 2014). It is a wind-pollinated, self-incompatible species with wind-dispersed seeds (Bonin et al. 2014). Herbarium records suggest that *M. sacchariflorus* first escaped domestic gardens sometime in the 1940s and 1950s, most likely along the Mississippi River in Iowa, Wisconsin, and Illinois, and feral populations have been reported in 11 states (Bonin et al. 2014; EDDMapS 2016). Most feral populations of *M. sacchariflorus* in North America are concentrated in the midwestern United States, whereas others occur in the northeastern United States and southeastern Canada (Bonin et al. 2014; EDDMapS 2016; Hager et al.

2015b). These escaped populations often form dense, monospecific stands along roadsides, ditches, and forest edges, typically in mesic habitats. The shoots are ~ 2 to 3 m tall with vigorous, laterally spreading rhizomes, which somewhat limits the utility of *M. sacchariflorus* as an ornamental plant. Currently, only a few cultivars of this clonally propagated ornamental are available commercially (see Discussion).

The potential spread and evolutionary trajectory of feral *M. sacchariflorus* in North America and elsewhere could be influenced by its ability to hybridize with closely related *Miscanthus* taxa that share the same number of chromosomes (Table 1). Native Asian populations of *M. sacchariflorus* can be diploid (with 38 chromosomes) or tetraploid (Chae et al. 2014; Glowacka et al. 2015; Sacks et al. 2013a), but the ploidy of feral populations in North America has not been reported. *Miscanthus sacchariflorus* is closely related to *Miscanthus sinensis* (Chinese silvergrass), which is a more popular diploid ($2N = 38$) ornamental that was introduced to the United States from Japan in the 1800s and has since become invasive (Quinn et al. 2012). In contrast to *M. sacchariflorus*, more than 50 cultivars of *M. sinensis* are available commercially, and feral populations have been reported in at least 26 states (EDDMapS 2016; Quinn et al. 2012). In their native ranges, *M. sacchariflorus* is endemic to more northern latitudes of East Asia (China, Korea, and Japan) than *M. sinensis*, although their ranges overlap (e.g., Schnitzler and Essl 2015). Distributions of feral populations of the two congeners in North America appear to reflect similar latitudinal differences, with *M. sacchariflorus* recorded at cooler and more northerly latitudes compared with *M. sinensis* (EDDMapS 2016; Hager et al. 2014; Schnitzler and Essl 2015). Although they rarely occur near each other as feral populations in the United States, hybridization between these taxa could potentially involve cultivated varieties that occur nearby and overlap in flowering times. Also, hybridization is known to have occurred between native populations of these species in Japan (Clark et al. 2015; Nishiwaki et al. 2011; Tamura et al. 2016).

Cultivars that might hybridize with *M. sacchariflorus* in the future may include new biofuel crops. Tetraploid *M. sacchariflorus* and diploid *M. sinensis* are the parent species of the triploid sterile hybrid *Miscanthus* \times *giganteus*, a bioenergy feedstock crop (Linde-Laursen 1993; Hodkinson et al. 2002a, 2002b; Smith and Barney 2014). Currently, a commercially developed fertile tetraploid (4x) form of *M. x giganteus* known as PowerCaneTM also is being considered as a bioenergy crop (Anderson et al. 2015; Sacks et al. 2013b). With a number of *Miscanthus* species now identified as promising candidates for cellulosic biofuel production, researchers are investigating whether some cultivars may escape cultivation and become invasive, hybridize with other feral or ornamental populations, or

Table 1. Potential gene flow networks for cultivated and feral populations of *Miscanthus* taxa based on similar ploidy levels. PowerCane is a fertile 4x hybrid between *M. sacchariflorus* and *M. sinensis* that has been developed for biofuel production. Hybridization between 2x and 4x plants typically result in sterile 3x hybrids. In this study, feral populations of *M. sacchariflorus* were 2x. See text for details and references.

	Cultivated populations	Feral populations
2x Potential gene flow network	2x <i>M. sacchariflorus</i> —ornamental 2x <i>M. sinensis</i> —ornamental	2x <i>M. sacchariflorus</i> (this study) 2x <i>M. sinensis</i> (well documented)
4x Potential gene flow network	4x <i>M. sacchariflorus</i> —ornamental 4x PowerCane—bioenergy cultivar	4x <i>M. sacchariflorus</i> (not found) 4x PowerCane (hypothetical)

both (e.g., Bonin et al. 2014; Hager et al. 2015a; Quinn et al. 2012; Smith and Barney 2014).

The goals of our current study of *M. sacchariflorus* in Iowa and Minnesota were to (1) examine the extent and partitioning of genetic diversity in feral populations, (2) gain a better understanding of the roles of clonal propagation and seed production as mechanisms for the spread of feral populations, and (3) determine whether feral populations include both 2x and 4x ploidy levels to understand their potential to hybridize with related taxa. *Miscanthus sacchariflorus* is thought to disperse and spread primarily by rhizomes because of reported low seed set (Bonin et al. 2014; Nishiwaki et al. 2011), but little is known about the ecological factors and plant traits that facilitate successful establishment of these feral populations. For instance, it is not clear whether feral populations exhibit genetic variation that could facilitate adaptation to local environments, whether cross-pollination has occurred within and among different cultivars, or whether some feral populations represent a single, clonally reproducing genotype, in which case self-incompatibility and spatial isolation could preclude seed set. Also, no data are yet available on ploidy levels within feral *M. sacchariflorus* populations, yet such information is critical for characterizing the potential for hybridization with closely related taxa, such as nonsterile *M. × giganteus*, PowerCane (Table 1). Ploidy also may provide insights about the origins of escaped cultivars, since diploid native populations of *M. sacchariflorus* have been reported in China and Korea, but not in Japan (Chae et al. 2014; Sacks et al. 2013a). These questions pertain to broader concerns about how easily feral *M. sacchariflorus* can continue to evolve and spread as a potentially invasive species.

Materials and Methods

Plant Populations. We focused on feral populations of *M. sacchariflorus* in two states in the U.S. Midwest, Iowa (IA) and Minnesota (MN). Feral populations of *M. sacchariflorus* were studied at a total of 22 sites in Iowa (10 counties) and six sites in Minnesota (two counties north of the Minneapolis/St.

Paul metropolitan area) in August and September 2013 (Table 2; Figure 1). We defined a population as a group of plants located at least 500 m away from other conspecifics. Distances between the sampled populations ranged from 6.6 km (IA120 vs. IA124) to 367.6 km (IA119 vs. IA126) in Iowa, and from 2.4 km (MN4 vs. MN5) to 16.4 km (MN3 vs. MN6) in Minnesota. Some populations might have become established directly from the extensive lateral spread of planted cultivars into adjacent areas (e.g., MN1, 2, and 6), but in all cases, the shoots appeared to be multiplying without cultivation. The populations were often made up of several spatially separated patches with no apparent rhizome connections to each other, with ~ 10 to > 100 shoots per patch (see photos in Bonin et al. 2014). We recorded the number of patches observed at each study population.

We also sampled three feral populations of *M. sinensis* in Ohio and West Virginia and an ornamental *M. sinensis* variety (cultivar ‘ZA274’, Jelitto Seed Company, www.jelitto.com) for ploidy analyses described below (Table 2). However, we were not able to include commercially available cultivars of ornamental *M. sacchariflorus* for comparisons with feral populations.

DNA sampling and amplification. DNA samples were collected from 15 populations of feral *M. sacchariflorus* in Iowa and six populations in Minnesota (Tables 2 and 3). Young leaf tissue was collected from 15 to 23 individual shoots per population, which were distributed among the patches, to represent the clonal diversity within each population. Samples were collected from shoots that were at least 5 m apart. Leaf tissue samples were individually labeled, stored in 2.0-ml microfuge tubes, and kept cool on ice until transported to the laboratory for storage at 4 C until DNA was extracted. In a similar manner, we collected leaf samples from 16 to 20 plants in each of three populations of feral *M. sinensis* in Ohio and West Virginia (Table 2) to serve as an out-group in the cluster analysis described below.

DNA was extracted from leaf samples using a modified filter paper method as in Adugna et al. (2011). We used eight expressed sequence tag-derived simple sequence repeats

Table 2. Sources of *Miscanthus* used in this study, including 28 feral populations of *M. sacchariflorus*, three feral populations of *M. sinensis*, and one cultivar of *M. sinensis*. Populations not included in SSR analyses are indicated by an asterisk. The cultivar of *M. sinensis* was used solely for ploidy comparisons.

Type	Origin	County	Population	Latitude °N	Longitude °W	Total no. of patches observed
Feral <i>M. sacchariflorus</i>	Iowa	Adams	IA119	40.9789	94.5671	9
		Boone	IA18	42.0979	93.8962	13
		Boone	IA91	41.8851	93.8969	8
		Boone	IA1*	41.9710	93.9220	6
		Dallas	IA40*	41.6884	94.0270	3
		Dallas	IA101*	41.6317	93.9796	5
		Dubuque	IA120	42.5471	90.8409	6
		Dubuque	IA124	42.4991	90.8879	4
		Dubuque	IA126	42.4962	90.6359	6
		Marshall	IA103	41.9931	93.1308	10
		Marshall	IA26	42.1225	93.0592	6
		Marshall	IA43*	41.9735	92.9075	12
		Muscatine	IA107	41.3455	91.1735	10
		Muscatine	IA85	41.5501	90.9005	9
		Muscatine	IA83*	41.5372	91.0377	4
		Muscatine	IA82*	41.4540	91.0255	2
		Scott	IA108	41.5965	90.7446	12
		Story	IA3*	41.9651	93.5521	1
		Story	IA102*	42.0226	93.3803	7
		Tama	IA118	41.9591	92.3414	12
	Washington	IA105	41.4682	91.8586	8	
	Washington	IA106	41.1921	91.5260	5	
	Minnesota	Ramsey	MN1	45.0099	93.0218	1
		Ramsey	MN2	44.9209	92.9944	1
		Ramsey	MN3	45.0168	92.9894	1
		Ramsey	MN5	45.0219	93.0802	3
		Ramsey	MN6	45.0279	93.1374	1
Washington		MN4	45.0279	92.9628	5	
Feral <i>M. sinensis</i>		Ohio	Washington	MSI1	39.4774	81.3011
	West Virginia	Wood	MSI2	39.2474	81.3594	—
		Wood	MSI3	39.3973	81.4364	—
Ornamental cultivar <i>M. sinensis</i>	Jelitto Seed Co., Germany	—	ZA274	—	—	—

(SSRs) (Supplemental Table S1) developed for *Miscanthus* by Kim et al. (2012). Two other SSR loci (MS 53, MS 29; Supplemental Table S1) were not used because they were not polymorphic in our samples from *M. sacchariflorus* or *M. sinensis*; likewise, 10 other SSR markers were not used because they were monomorphic in samples from both species. Because we did not use the monomorphic markers in reporting genetic diversity, these metrics should be interpreted accordingly. We also note that the number of distinct clones reported in each population could have been underestimated by using only eight SSR loci.

The forward primers were labeled with fluorescent dyes (6-FAM, HEX, or NED; Invitrogen™, Thermo Fisher

Scientific, Waltham, MA). Polymerase chain reaction (PCR) was performed in 10 µl of final volume containing 5 µl of 2× Qiagen® Multiplex PCR Master Mix (Applied Biosystems, Inc., Foster City, CA), 1 µl of 5 mM primer mix (containing 3 or 4 primers each at 5 mM; Invitrogen), and 4 µl of template DNA. The PCR program consisted of 95 C for 15 min, followed by 11 touchdown cycles at 95 C for 30 s, 63 C (−1 C cycle^{−1}) for 30 s, and 72 C for 60 s, followed immediately by 40 cycles at 95 C for 30 s, 57 C for 60 s, 72 C for 60 s, and a final extension step of 15 min at 72 C. After PCR, 1 µl of the product was combined with 0.8 µl of a universal ROX-labeled internal standard (DeWoody et al. 2004) and 8.2 µl of HiDi formamide

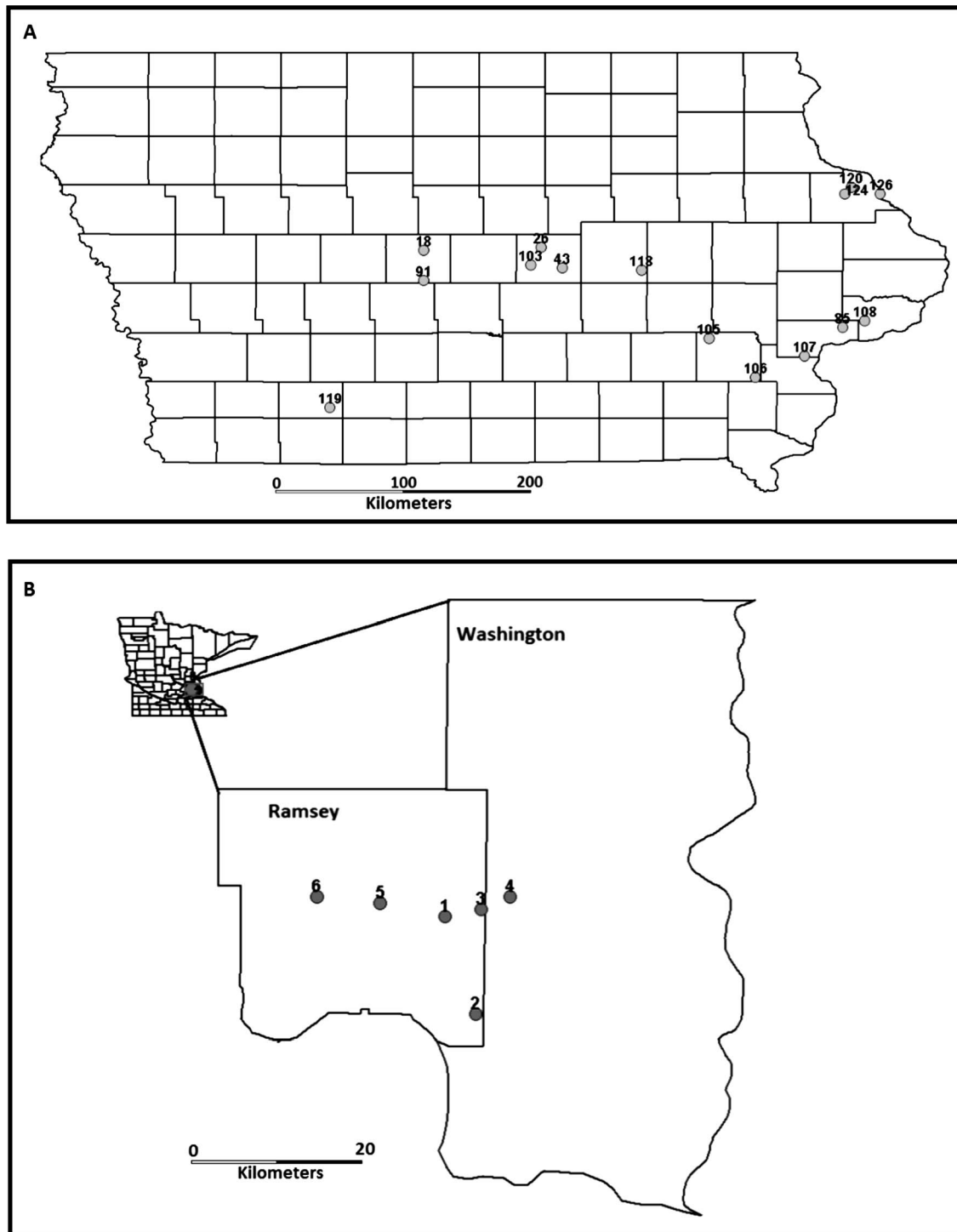


Figure 1. Map showing collection sites in (A) Iowa (15 populations, nine counties) and (B) Minnesota (six populations, two counties) for the 21 feral *M. sacchariflorus* populations that were genotyped using SSR markers. See Table 2 for details.

(Applied Biosystems) for fragment size analysis on an ABI Prism 3100 genetic analyzer (Applied Biosystems). Peak detection and allele calling was performed using the software GeneMapper 3.7 (Applied Biosystems).

Population Genetic Analysis. The software GenClone 2.0 was used to identify the number of multilocus genotypes (MLGs) and to estimate the extent of genotypic diversity for each population (Arnaud-Haond and Belkhir 2007). The

Table 3. Clonal and genetic diversity measures^a based on eight polymorphic SSR loci for 21 feral populations of *Miscanthus sacchariflorus* in Iowa and Minnesota.

Population	<i>N</i>	No. of patches sampled ^b	MLGs ^c	R	<i>N_a</i>	<i>D</i> [*]	ED [*]	<i>H_e</i>	<i>H_o</i>	<i>F_{IS}</i>
IA103	18	5 (10)	4 (4)	0.18	1.63	0.59	0.59	0.31	0.50	0.03
IA105	20	8 (8)	2 (1)	0.05	1.63	0.34	0.56	0.56	1.00	-0.78
IA106	23	5 (5)	3 (2)	0.09	1.75	0.31	0.27	0.36	0.64	-0.24
IA107	15	7 (10)	4 (4)	0.21	1.88	0.60	0.53	0.34	0.53	0.00
IA108	15	10 (12)	4 (3)	0.21	1.88	0.67	0.68	0.37	0.56	-0.09
IA118	15	11 (12)	6 (5)	0.36	2.00	0.85	0.86	0.44	0.60	-0.17
IA119	17	7 (9)	4 (3)	0.19	1.88	0.64	0.66	0.38	0.57	-0.09
IA120	18	6 (6)	6 (5)	0.29	2.00	0.62	0.33	0.47	0.71	-0.33
IA124	23	4 (4)	3 (3)	0.10	1.88	0.26	0.15	0.37	0.64	-0.23
IA126	21	5 (6)	3 (2)	0.10	1.63	0.19	0.00	0.36	0.61	-0.13
IA18	16	8 (13)	3 (2)	0.13	1.75	0.49	0.53	0.52	0.88	-0.51
IA26	22	8 (8)	3 (2)	0.10	1.88	0.18	0.00	0.42	0.75	-0.38
IA43	20	11 (12)	2 (1)	0.05	1.75	0.19	0.21	0.43	0.74	-0.34
IA85	15	6 (9)	3 (2)	0.14	1.88	0.53	0.60	0.42	0.66	-0.27
IA91	23	5 (8)	3 (2)	0.09	1.63	0.17	0.00	0.35	0.63	-0.15
Mean				0.15	1.80	0.44	0.40	0.41	0.67	-0.25
MN1	19	1 (1)	5 (5)	0.22	1.88	0.62	0.51	0.44	0.67	-0.27
MN2	20	1 (1)	6 (5)	0.26	2.00	0.71	0.60	0.44	0.70	-0.39
MN3	20	1 (1)	4 (3)	0.16	1.88	0.43	0.29	0.37	0.64	-0.23
MN4	17	3 (5)	5 (5)	0.25	2.25	0.66	0.56	0.43	0.70	-0.44
MN5	17	3 (3)	5 (4)	0.19	1.88	0.64	0.66	0.42	0.69	-0.34
MN6	16	1 (1)	3 (3)	0.13	1.88	0.34	0.21	0.41	0.64	-0.20
Mean				0.20	1.96	0.57	0.47	0.42	0.67	-0.31

^a Abbreviations: *N*, number of samples; MLGs, number of multilocus genotypes; R, clonal richness, a weighted measure of MLGs per population; *N_a*, average number of alleles per locus; *D*^{*}, Simpson complement, a measure of genotypic diversity; ED^{*}, Simpson evenness, a measure of genotypic heterogeneity; *H_e* and *H_o*, expected and observed heterogeneity, respectively; *F_{IS}*, fixation index.

^b Number of patches sampled; includes the total number of observed patches in parentheses.

^c Conservative estimates of the numbers of MLGs in each population are listed in parentheses, after deleting those that differed at only one locus and therefore might be different because of a somatic mutation.

genotypic richness index, R, estimates the proportion of distinct MLGs in a population relative to the number of sampling units and is calculated as $R = (G - 1)/(N - 1)$, where *G* is the number of multilocus genotypes or lineages detected in a sample, and *N* is the sample size (Dorken and Eckert 2001). Values of R range between 0, indicating monoclonality, and 1, indicating that all genotypes in a sample are unique. The parameter *D*^{*} is a modified complement of the widely used Simpson index (Simpson 1949). It describes the probability that two randomly sampled individuals represent different clonal lineages (Arnaud-Haond et al. 2007). Simpson's evenness index (ED^{*}), is a measure of genotypic heterogeneity; it estimates how equitably the MLGs are distributed among individuals within populations (Arnaud-Haond et al., 2007). GenClone 2.0 was also used to calculate observed heterozygosity (*H_o*), unbiased expected heterozygosity (*H_e*) (Nei 1978), and the

fixation index (*F_{IS}*) based on a round robin resampling procedure to account for rare alleles (Parks and Werth 1993). The average number of alleles per locus (*N_a*) was calculated using the software GenAIEEx 6.501 (Peakall and Smouse 2012).

Because *M. sacchariflorus* has the capacity for both clonal and sexual reproduction, individuals with identical MLGs could be members of the same clone or could arise by chance from independent sexual events. The probability of the latter was calculated for MLGs that were detected more than once in the same population based on the method of Parks and Werth (1993) implemented in the program MLGsim 2.0 (Stenberg et al. 2003). MLGsim was used to compute *P_{sex}* values, which show the likelihood that a set of repeated MLGs in a population originate from sexual reproduction under random mating. We tested the significance of the observed values based on P values generated from the distributions of 10⁴ simulated *P_{sex}*

values at each population. A critical P value of 0.05 was adopted, below which a set of repeated multilocus genotypes was considered to be clonal.

The genetic structure of feral *M. sacchariflorus* was investigated by performing an analysis of molecular variance (AMOVA) using GenALEx 6.5 (Peakall and Smouse 2012). A hierarchical cluster analysis also was performed to visualize genetic relationship patterns within and among populations of *M. sacchariflorus* in the two states, with three feral populations of *M. sinensis* serving as an out-group. A matrix of pairwise Nei's genetic distances (Nei 1972) among the populations was generated in GenALEx 6.5, and imported into DARwin 6.0 (DARwin software version 6.501. CIRAD Department Systèmes Biologiques (BIOS), Avenue Agropolis—TA A75/02 34398 Montpellier Cedex 5—FRANCE) for cluster dendrogram construction based on the unweighted pair group method with arithmetic mean (UPGMA).

Seed Production. Each shoot produces a single large panicle. To quantify relative levels of seed production in feral *M. sacchariflorus*, we collected 10 to 99 mature panicles representing different patches from each of 14 populations in Iowa (with 2 to 15 patches) and six populations in Minnesota (with one to five patches) in September 2013 before seed dispersal. Panicles from each population were pooled in a single paper bag. Panicles were hand-threshed in the laboratory, and seeds were counted, including any that had come off in the bag. Very few seeds were found, so it was feasible to count each individual seed. Seed set was recorded as the number of seeds per panicle for each population (i.e., the number of seeds divided by the number of panicles that were sampled).

We hypothesized that the very low seed production we had observed previously might be because of a lack of outcross pollen in this clonal, self-incompatible species. If so, we expected that seed production would be highest in populations with the greatest number of MLGs. For 12 populations from which we had collected at least 20 panicles and DNA for MLG analyses, we used Pearson's correlation with the software R (R Core Team, 2015) to test for positive correlations between the average number of seeds per panicle and (1) the number of MLGs, (2) R (a measure of clonal richness), and (3) the number of patches.

Ploidy Analysis. We collected rhizomes from 11 representative populations in Iowa to determine whether feral *M. sacchariflorus* was 2x or 4x, given that both types of ornamental cultivars have been reported (Chae et al. 2014). To obtain leaf tissues, rhizome fragments approximately 10 cm long, each with four to six nodes, were excavated from three or four shoots from different patches in each

population. Rhizomes were rinsed with water to remove excess soil, wrapped in moist paper towels, and placed in plastic bags. Samples were transported on ice to the laboratory and kept refrigerated at 4 C until planted in a greenhouse to produce leaf tissue. In a similar manner, we sampled three or four plants from each of three feral *M. sinensis* populations from West Virginia and Ohio and four individuals of ornamental *M. sinensis* grown from seed (Table 2).

Flow cytometry was used to estimate relative DNA content to infer ploidy levels following the method of Galbraith et al. (1983). Approximately 2 cm² of fresh leaf tissue for each sample was chopped with a razor blade in a petri dish containing 0.5 ml of a prechilled lysis buffer that consisted of 45 mM magnesium chloride, 30 mM sodium citrate, 20 mM MOPS [3-(*N*-morpholino)propanesulfonic acid], and 0.1% Triton X-100 at pH 7 (Galbraith et al. 1983) (Sigma-Aldrich Co., St. Louis, MO). The cell suspension was filtered through a 30- μ m filter (CellTrics[®] 30 μ m, Sysmex North America Inc., Lincolnshire, IL) and centrifuged at 2,000 rpm for 5 min to pellet the cells. The supernatant was discarded, and the pellet was resuspended in 0.5 ml of the lysis buffer before adding 0.3 ml of propidium iodide to stain the nuclei (Sigma-Aldrich Co.). We included an equal amount of leaf tissue from a sterile, triploid *M. × giganteus* 'Illinois' individual in each sample as an internal standard, as in Chae et al. (2014). The stained suspension was processed through a flow cytometer (BD[™] LSR II; BD Biosciences, San Jose, CA) to determine the mean sample nucleus fluorescence relative to that of the internal standard. Genome sizes were estimated based on a minimum of 3,000 nuclei. Nuclear DNA content for unreduced tissue (2C) was calculated using the equation: sample 2C DNA content = (mean sample nuclei fluorescence \times standard 2C DNA content) / mean standard nucleus fluorescence value.

Results

Population Genetic Analyses. For the eight polymorphic SSR loci, the average number of alleles per locus across the feral *M. sacchariflorus* populations ranged from 1.63 to 2.25 (Table 3; mean = 1.85). Allele number and other measures of diversity were somewhat higher for the five Minnesota populations compared with the 15 Iowa populations. In both states, the average observed heterozygosity was 0.67, whereas average expected heterozygosity was 0.41 or 0.42 (Table 3). All populations except two (IA103; F_{IS} = 0.03 and IA107; F_{IS} = 0.00) had negative fixation indices (Table 3; average = -0.27), which indicates excess heterozygosity.

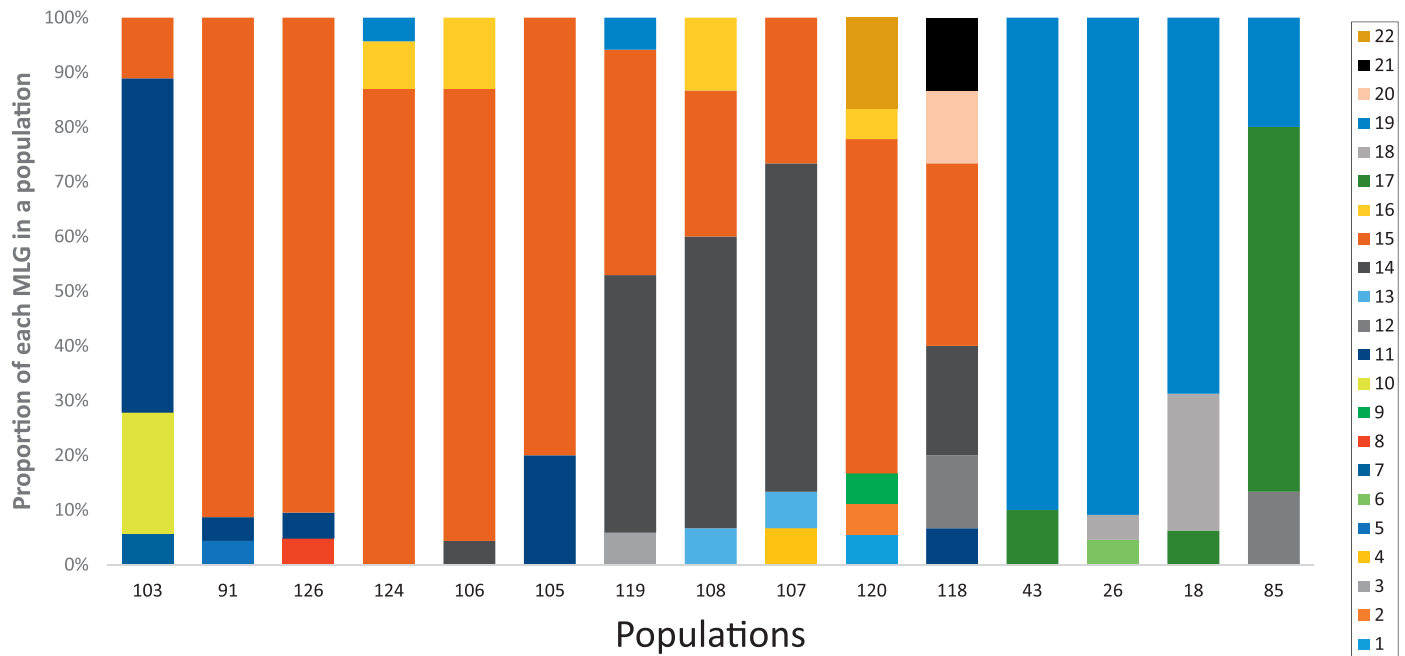


Figure 2. Distribution of 22 multilocus genotypes (MLGs) detected in 15 feral populations of *Miscanthus sacchariflorus* from Iowa. See Table 3 for sample sizes and Supplemental Figure S1 for the distribution of all 34 MLGs in Iowa and Minnesota.

Across all 21 populations of feral *M. sacchariflorus* ($N = 390$), we found only 34 distinct MLGs, many of which were observed repeatedly within and among populations (Table 3; Figure 2; Supplemental Figure S1). Close to 40% of the MLGs were represented in more than one population across the sampled range, and one MLG (labeled MLG N in Supplemental Figure S1) was shared between states. Within populations, most MLGs appear to be clonal replicates, rather than the result of sexual reproduction, because 80% (37 of 46 of the MLG replicates in Table 4) had observed P_{sex} values < 0.01 and were significant at $P \leq 0.05$, falling outside distribution of simulated values for random mating (Table 4). Nonsignificant ($P > 0.05$) P_{sex} values were observed for nine MLGs in seven populations, suggesting that sexual reproduction events may be responsible for some repeated occurrences of these MLGs.

Clonal richness (R) measures the diversity of clones within populations, with 0.0 representing a single clone. R averaged only 0.15 (range 0.05 to 0.36) and 0.20 (range 0.13 to 0.26) in Iowa and Minnesota, respectively, and was 0.08 for all populations combined ($N = 390$). Simpson's complement (D^*) values were also generally low, averaging 0.44 among populations in Iowa and 0.57 among their counterparts in Minnesota, pointing to low levels of genetic variation. Mean Simpson's evenness, ED^* , a measure of equitability in the distribution of the scored MLGs, was 0.41 across all *M. sacchariflorus* populations, indicating that on average they are dominated by one or a few highly represented MLGs (Figure 2; Supplemental Figure S1).

The feral populations were not highly differentiated based on AMOVA, which showed that 8% of the total genetic variation was explained by differences between states and 90% was partitioned within populations (Table 5). Within states, we found low but statistically significant differentiation in *M. sacchariflorus* from Iowa, with 3% of the total genetic variation explained by differences among populations and a corresponding F_{st} value of 0.034 ($P = 0.002$). For *M. sacchariflorus* in Minnesota, 100% of the total genetic variation was partitioned within populations with a corresponding F_{st} value of -0.016 ($P = 0.89$), indicating no evidence of differentiation among the six populations. Likewise, cluster analysis based on pairwise Nei's genetic distance and UPGMA (Figure 3) assigned the 21 *M. sacchariflorus* populations to two clusters, with *M. sinensis* as a very distinct out-group. Populations from Iowa were represented in each of the two major clusters, whereas the Minnesota counterparts were assigned in only one of the two major clusters, where they occurred as an exclusive subgroup.

To summarize, our results show that a low level of genetic separation exists between the feral populations from the two states. Cluster analysis revealed generally short branching lengths within the two major clusters of *M. sacchariflorus*, indicating that the feral populations did not differ greatly from each other. This outcome is in accordance with our results showing that a substantial number of MLGs (40%) in the *M. sacchariflorus* pool were shared among the feral populations.

Table 4. P_{sex} values and corresponding levels of significance for each set of replicated multilocus genotypes (MLGs) in 21 feral populations of *Miscanthus sacchariflorus* in Iowa and Minnesota. All but nine MLGs had significant P_{sex} values and were therefore likely to represent clonal replicates. MLGs that were represented only once in a population are not included in this table.

Population	MLG	<i>N</i>	P_{sex}	P Value	Level ^a	Population	MLG	<i>N</i>	P_{sex}	P value	Level ^a	
IA105	1	4	9.3E-04	0.027	*	IA126	1	19	0.0E+00	0.000	**	
	2	16	4.4E-16	0.000	**		IA18	1	4	3.5E-05	0.015	*
IA103	1	4	5.7E-03	0.092	ns	IA26	2	11	0.0E+00	0.000	**	
	2	11	2.0E-10	0.000	**		IA43	1	20	0.0E+00	0.000	**
	3	2	5.5E-03	0.089	ns		IA85	1	2	1.5E-02	0.903	ns
IA106	1	19	0.0E+00	0.000	**	IA85	2	18	6.7E-16	0.000	**	
	2	3	9.2E-05	0.017	*		IA91	1	2	4.2E-04	0.095	ns
IA107	1	9	1.2E-09	0.000	**	IA91	2	10	6.1E-14	0.000	**	
	2	4	3.5E-05	0.002	**		MN1	3	3	4.9E-05	0.018	*
IA108	1	8	5.8E-09	0.000	**	MN1	1	21	0.0E+00	0.000	**	
	2	4	9.2E-05	0.006	**		2	5	5.5E-07	0.000	**	
	3	2	5.9E-04	0.066	ns		2	11	8.9E-16	0.000	**	
IA118	1	2	1.4E-03	0.548	ns	MN2	1	10	6.0E-14	0.000	**	
	2	3	4.5E-04	0.401	ns		2	2	3.4E-04	0.138	ns	
	3	5	6.6E-08	0.000	**		3	5	5.6E-07	0.002	**	
	4	2	1.2E-05	0.023	*		MN3	1	3	7.5E-05	0.018	*
	5	2	1.2E-05	0.023	*			2	15	1.0E-15	0.000	**
IA119	1	8	1.1E-08	0.000	**	MN4	1	5	7.7E-08	0.003	**	
	2	7	1.8E-08	0.000	**		2	9	1.2E-12	0.000	**	
IA120	1	11	0.0E+00	0.000	**	MN5	1	7	2.6E-10	0.000	**	
	2	3	4.3E-08	0.000	**		2	8	1.6E-10	0.000	**	
IA124	1	20	0.0E+00	0.000	**	MN6	1	13	0.0E+00	0.000	**	
	2	2	1.5E-03	0.072	ns		2	2	2.5E-05	0.002	**	

^a Abbreviations: IA, Iowa; MN, Minnesota; ns, not significant.

* Value is significant at $P < 0.05$.

** Value is significant at $P < 0.001$.

Table 5. Analysis of molecular variance^a for feral populations of *Miscanthus sacchariflorus* in Iowa and Minnesota based on (A) both states and (B) each state analyzed separately.

Source	df	SS	MS	%	F_{st}	P value
A.						
Between states	1	40.942	40.942	8	0.078	0.001
Among pops	19	48.088	2.531	2		
Within pops	759	1071.297	1.411	90		
Total	779	1,160.327		100		
B.						
Iowa						
Among pops	14	44.802	3.200	3	0.034	0.002
Within pops	547	753.294	1.377	97		
Total	561	798.096		100		
Minnesota						
Among pops	5	3.287	0.657	0	-0.016	0.890
Within pops	212	318.002	1.500	100		
Total	217	321.289		100		

^a Abbreviations: Pops, populations; MS, mean sum of squares; SS, sum of squares; F_{st} , fixation index.

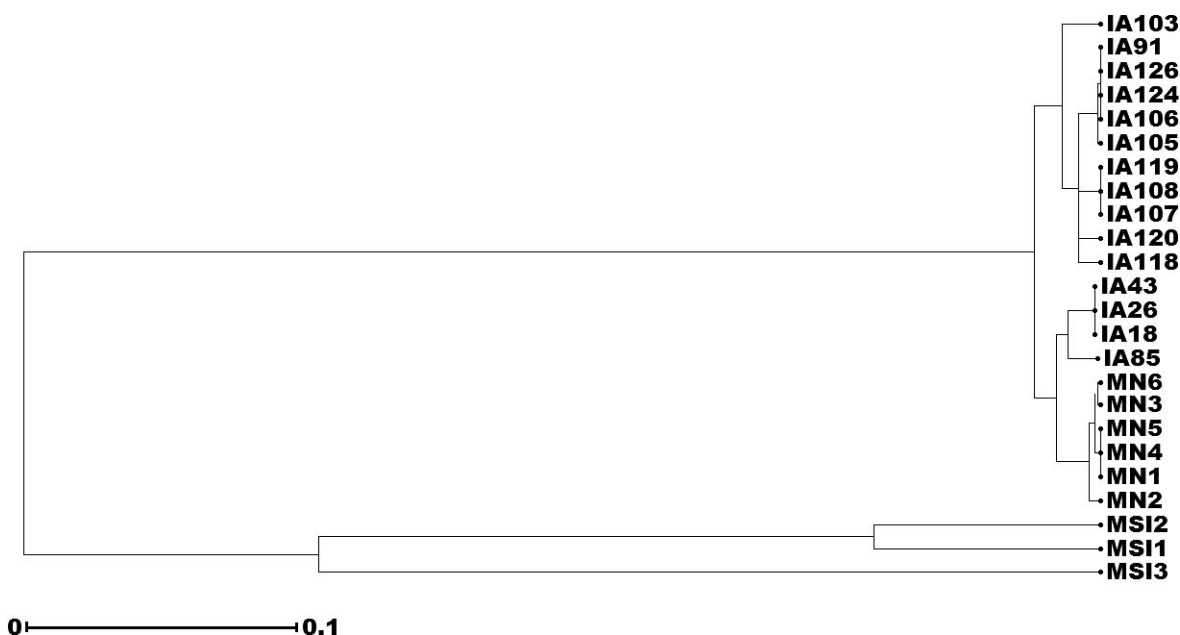


Figure 3. Cluster analysis based on the unweighted pair group method with arithmetic mean (UPGMA) of pairwise Nei's (1978) genetic distance among the 21 feral populations of *M. sacchariflorus* in Iowa and Minnesota, with three feral *M. sinensis* populations included as an out-group (labeled MS1-3). See Table 2 and Figure 1 for locations of these populations.

Multilocus Genotypes, Clonal Structure, and Seed Set.

Seed set in *M. sacchariflorus* requires outcross pollen from another clone. Despite extensive clonality and rather limited sample sizes, we found more than one MLG in each of the 21 populations that were analyzed for SSR diversity ($N = 15$ to 23 samples per population; Table 3). Also, for patches that were sampled more than once within populations, 49% had more than one MLG (out of 80 patches). The greatest number of MLGs detected per patch was four (in IA120 and MN4), and the greatest number per population was six, found in IA118 and MN6. Within each population, nearly all of the MLGs differed from each other at more than one locus (Tables 3 and 6), suggesting that each MLG was a different individual rather than a somatic mutant of the same individual. It is possible that more extensive sampling would have revealed additional MLGs in some patches and populations.

On the basis of MLG data, our results indicate that nonself pollen sources were present for pollinating clones within all populations and even within many patches within populations. Nonetheless, every feral population sampled had very poor seed set (Table 6). The average numbers of seeds found per panicle ranged from none for populations at MN2 and MN3, to a maximum of 0.30 at IA103 and 0.27 at MN5. Most populations had < 0.04 seeds per panicle. For the populations that also were analyzed for MLGs, the correlations between seed set and number of MLGs, R, or number of patches were not

statistically significant ($P > 0.10$; $N = 12$). However, we note that multiple MLGs were detected in the two populations with relatively greater seed set. IA103 had five patches, each of which included at least two MLGs, and MN5 had three patches, each of which included 2 or 3 MLGs.

Ploidy. All of the 11 sampled populations of feral *M. sacchariflorus* were diploid, with average DNA content ranging from 4.3 to 4.4 pg (Table 7). The feral and cultivar *M. sinensis* samples in our analysis (listed in Table 2) were confirmed as diploid (2C DNA content: 5.1 to 5.3 pg). Rayburn et al. (2009) also reported genome sizes of 4.5 pg for diploid *M. sacchariflorus* ('Robustus' cultivar) and 5.5 pg for diploid *M. sinensis*.

Discussion

Inferences from Population Genetics. Overall, we found that feral populations of *M. sacchariflorus* in Iowa and Minnesota are very similar genetically and represent a limited number of multilocus genotypes (only 34 MLGs from 390 samples). Theoretically, multiple occurrences of the same MLG could be due to sexual reproduction among genetically similar individuals. However, we assume that individuals with the same MLG for the eight polymorphic SSR markers are mainly, but not exclusively, the result of

Table 6. Number of seeds per panicle, number of observed patches, and number of multilocus genotypes (MLGs) detected in feral populations of *Miscanthus sacchariflorus* in Iowa (IA) and Minnesota (MN). MLGs are unique based on 15–23 DNA samples per population; see Tables 2 and 3 for details. Six of the populations listed below were not screened for MLGs.

Population	No. of seeds per panicle	Total no. of patches observed	No. of MLGs detected ^a	Total no. of panicles sampled	Total no. of seeds
Iowa					
IA1	0.08	6	—	13	1
IA3	0.02	1	—	49	1
IA18	0.05	15	3 (2)	78	4
IA43	0.01	12	2 (1)	68	1
IA82	0.02	2	—	60	1
IA83	0.13	4	—	52	7
IA85	0.01	9	3 (2)	69	1
IA91	0.07	8	3 (2)	70	5
IA101	0.02	5	—	62	1
IA102	0.05	7	—	60	3
IA103	0.30	10	4 (4)	99	30
IA118	0.05	12	6 (5)	59	3
IA120	0.02	6	6 (5)	52	1
IA126	0.07	6	3 (2)	61	4
Minnesota					
MN1	0.10	1	5 (5)	20	2
MN2 ^b	0.00	1	6 (5)	10	0
MN3 ^b	0.00	1	4 (3)	10	0
MN4	0.08	5	5 (5)	50	4
MN5	0.27	3	5 (4)	30	8
MN6	0.10	1	3 (2)	20	2

^a Conservative estimate of the number of MLGs in each population listed in parentheses after deleting those that differed at only one locus and therefore might be different due to a somatic mutation.

^b Note the small sample size for these two populations (10 panicles).

vegetative propagation via rhizomes, as supported by P_{sex} analyses (Table 4).

Because the feral populations in our study were diploid rather than tetraploid (Table 7), they are more likely to have come from native populations in China or Korea than Japan, based on surveys carried out by Sacks et al. (2013a) and Chae et al. (2014). Also, the very low number of MLGs that we observed per population (Table 3) suggests that a few very similar cultivars may have been involved in the naturalization process. Chae et al. (2014) compared genetic relationships among three ornamental cultivars of *M. sacchariflorus*, including Robustus, using 17 SSR markers and found them to be extremely similar, with two appearing to be undifferentiated from each other. Deciphering the cultivated origins of feral populations is complicated by our observation that homeowners have been observed collecting rhizomes of *M. sacchariflorus* from feral populations, including MN4, to plant as ornamentals. Likewise, a property owner for population IA101 in Iowa, which was not genotyped in the current study, mentioned having collected plants directly from feral plants growing in

a ditch. Another property owner told us that population IA124 began as a planted population that was partly from nursery stock and partly from feral populations collected from unknown locations. Thus, we cannot assume that all *M. sacchariflorus* used for home landscaping purposes originated directly from commercial cultivars.

Commercial nurseries typically propagate ornamental grasses vegetatively, and the number of distinct varieties of *M. sacchariflorus* that are currently available in North America appears to be small. For example, Earthly Pursuits (2901 Kuntz Road, Winsor, MD) sells only two varieties of *M. sacchariflorus*. One is Robustus, or giant silver banner grass, and the other is known as Japanese silver banner grass. Additionally, Hortico Nursery (422 Concession 5 Road, Waterdown, ON, Canada) sells only one variety, known as silver banner grass. In contrast, more than 50 varieties of *M. sinensis* are available at these nurseries and elsewhere (Quinn et al. 2012). Although other ornamental varieties of *M. sacchariflorus* have probably been available since the early 1900s, *M. sacchariflorus* does not appear to have been very widely used as a landscaping plant.

Table 7. Nuclear DNA content (mean \pm SE) and presumed ploidy of feral *Miscanthus sacchariflorus* from Iowa (IA), feral *Miscanthus sinensis* (MSI) from Ohio (OH) and West Virginia (WV), and an ornamental cultivar of *M. sinensis*.

Type	Population	2C nuclear DNA content (pg) ^a	Presumed ploidy	
Feral <i>M. sacchariflorus</i>	IA18	4.3 \pm 0.07 ($n = 4$)	2x	
	IA26	4.3 \pm 0.03 ($n = 4$)	2x	
	IA40	4.3 \pm 0.01 ($n = 4$)	2x	
	IA43	4.4 \pm 0.07 ($n = 4$)	2x	
	IA91	4.3 \pm 0.05 ($n = 3$)	2x	
	IA101	4.4 \pm 0.03 ($n = 3$)	2x	
	IA103	4.4 \pm 0.03 ($n = 4$)	2x	
	IA106	4.3 \pm 0.02 ($n = 4$)	2x	
	IA108	4.3 \pm 0.04 ($n = 4$)	2x	
	IA118	4.4 \pm 0.04 ($n = 4$)	2x	
	IA124	4.4 \pm 0.06 ($n = 3$)	2x	
	Feral <i>M. sinensis</i>	MSI1, OH	5.3 \pm 0.01 ($n = 4$)	2x
		MSI2, WV	5.1 \pm 0.08 ($n = 3$)	2x
MSI3, WV		5.3 \pm 0.03 ($n = 4$)	2x	
Ornamental cultivar of <i>M. sinensis</i>	ZA274, ^b Jelitto Seed Co.	5.3 \pm 0.03 ($n = 4$)	2x	

^a Genome content was calculated using sterile *Miscanthus* \times *giganteus* 'Illinois' as an internal standard (2C = 6.86 pg), as in Chae et al. (2014).

^b Cultivar ID number.

However, the cold hardiness of *M. sacchariflorus* surpasses that of *M. sinensis* (e.g., Yan et al. 2012), making this plant attractive for colder climates. In the Minneapolis–St. Paul metro area, we have observed ornamental *M. sacchariflorus* more frequently than *M. sinensis*.

In summary, it seems likely that the feral populations in our study originated from a few commercially available cultivars that have hybridized to produce a limited set of MLGs derived from seeds. The 15 feral populations sampled in Iowa may have originated from a wider varietal range of ornamental *M. sacchariflorus* than the six populations sampled in Minnesota, which were very closely related and had fewer MLGs. Some populations may represent introductions from multiple cultivars or their feral progeny. For example, both IA118 and MN2 were found to have six MLGs that were fairly evenly distributed among the assayed individuals. Multiple introductions from different sources may facilitate crossing among compatible mates, as indicated by successful, albeit very poor, seed set in most of sampled populations.

Despite having a limited number of MLGs, feral populations of *M. sacchariflorus* in the current study had high levels of heterozygosity for the polymorphic loci that we examined. The negative fixation index values (Table 3) point to excess heterozygosity, probably resulting from outcrossing due to self-incompatibility and asexual reproduction of heterozygous clones, similar to other highly clonal species (Balloux et al. 2003; Gitzendanner et al. 2012; Gustafson et al. 2013; Lin et al. 2009).

Low Seed Set. Average seed set in the study populations varied from zero to only 0.3 seeds per panicle, consistent with our previous observations and reports on the internet (e.g., <http://miscanthus.cfans.umn.edu/sacchariflorus.html>). Each panicle produced hundreds of florets, and such low levels of seed production are puzzling. The few seeds we recovered were germinable (data not shown), and we assume that the timing of seed collection was neither too early nor too late in the season. Initially, we hypothesized that seed set was limited by a lack of sexually compatible pollen from flowering shoots in the vicinity of each MLG. However, each population had more than one MLG, as did many patches within populations. This suggests that compatible mates were available, but pollen limitation still may be a factor given that most populations were rather small and isolated. Another contributing mechanism for low seed set could be earlier deposition of self-incompatible pollen that interfered with the success of compatible pollen on the plants' stigmas.

Poor seed set has previously been reported in nursery-grown *M. sacchariflorus* in the United States (Meyer and Tchida 1999), and Hager et al. (2015b) found no evidence of a germinable seed bank in a survey of escaped populations of the species in Canada. Moreover, low pollen production has been noted in a single U.S. cultivar of *M. sacchariflorus* during breeding studies (E. Sacks, University of Illinois, personal communication to A. Snow). However, in Japan, a native tetraploid populations

of *M. sacchariflorus* had ~ 10 to 50% seed set (Nishiwaki et al. 2011), which is much greater than found in the current study. In North American feral populations, further research is needed to examine pollen production and to determine whether seed set is limited by the availability of compatible pollen, low pollen viability, or both. Over time, we hypothesize that the accumulation of diverse genotypes with sufficient pollen production might enable populations to produce more seeds and perhaps become more invasive. Currently, our findings are consistent with the conclusions of Hager et al. (2015b), who argued that the major mechanism by which *M. sacchariflorus* disperses along roadways and drainage ditches is by means of vegetative fragments.

Potential for Hybridization. Hybridization with other taxa is another mechanism by which feral populations could produce seeds and perhaps benefit from heterosis or other traits that enhance invasiveness (e.g., Hovick and Whitney 2014). This scenario seems possible under some circumstances involving hybrids between diploid feral *M. sacchariflorus* and cultivars of diploid *M. sinensis*. Accessions of both species from Asia show a wide range of flowering times that overlap in common garden experiments (Jensen et al. 2011; Yan et al. 2012) and in some native habitats where they have hybridized (Clark et al. 2015; Nishiwaki et al. 2011). In North America, geographic distributions of feral populations of these two species rarely overlap (e.g., Schnitzler and Essl 2015), but ornamental varieties of *M. sinensis* are widely planted in some regions where both cultivated and feral *M. sacchariflorus* occur, such as in Iowa. This situation could allow interspecific cross-pollination, especially involving the earliest flowering and most cold tolerant varieties of *M. sinensis*. As for new biofuel cultivars, neither the sterile 3x nor the fertile 4x (PowerCane) cultivars are expected to produce fertile offspring by hybridizing with feral 2x populations of *M. sacchariflorus*. Whether or not most feral populations of *M. sacchariflorus* in North America are diploid should be confirmed with more extensive sampling.

Potential for Invasiveness. The well-known lag time that can occur before an introduced species becomes invasive may or may not be taking place in feral *M. sacchariflorus*, but there is reason to be cautious about the future invasiveness of this species. In the self-incompatible Callery pear (*Pyrus calleryana* Decne.), for example, seed production was low for many years until multiple sexually compatible clones were commercialized, thereby facilitating the rapid local proliferation of feral populations (Culley and Hardiman 2007; Hardiman and Culley 2010).

Likewise, another introduced species, reed canarygrass (*Phalaris arundinacea* L.) appears to have become more genetically diverse and vegetatively prolific after multiple introductions from different sources (Lavergne and Molofsky 2007). Such intraspecific crossing between previously isolated populations may contribute to invasiveness via heterosis, greater genetic diversity, or both (e.g., Hovick and Whitney 2014). Even in the absence of intraspecific hybridization and genetic variation, introduced species are sometimes invasive. For example, an invasive perennial grass with a cosmopolitan distribution, *Arundo donax* L., also known as giant reed, represents only one or a few genetic clones that are typically sterile, yet it disperses very effectively via rhizome fragments (Ahmad et al. 2008; Pilu et al. 2014).

Here, we suspect that clonal growth, a dearth of MLGs, and isolation of many patches may have contributed to pollen-limited seed set in these feral populations of *M. sacchariflorus*. With this in mind, warnings about the invasive potential of *M. sacchariflorus* should be carefully considered, given that it already forms dense monospecific stands that reduce local biodiversity (e.g., Hager et al. 2015b). The Minnesota Department of Natural Resources (2016) notes that *M. sacchariflorus* is “not a severe threat at this time . . . [but] it should be monitored and eliminated in the open landscape.” This recommendation is consistent with common wisdom that the most effective way to manage invasive species is to limit their spread before rather than after they have become ubiquitous.

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