Winter hardiness of *Miscanthus* (II): Genetic mapping for overwintering ability and adaptation traits in three interconnected *Miscanthus* populations

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Abstract

*Miscanthus × giganteus* (M×g) is the primary species of *Miscanthus* for bioenergy feedstock production. The current leading biomass cultivar, M×g ‘1993-1780’, is insufficiently adapted in temperate regions with cold winters such as USDA hardiness zone 5 (average annual minimum temperature of −28.9 to −23.3°C) or lower. Three interconnected *Miscanthus* F₁ populations that shared a common parent were planted in a replicated field trial at Urbana, IL (hardiness zone 5b; average annual minimum temperature of −26.1 to −23.3°C) in spring 2011. The winter of 2013–2014 was especially cold in Urbana, with a minimum soil temperature at 10 cm of −6.2°C and a minimum air temperature of −25.3°C, giving us an opportunity to evaluate hardiness on established year-3 plants. The parent in common to all three populations, *M. sinensis* ssp. *condensatus* ‘Cosmopolitan’, is native to maritime southern Japan, and in Urbana, it is winter-damaged most years. In contrast, the three other parents, *M. sacchariflorus* ‘Robustus’ (MapA), *M. sinensis* ‘Silberturm’ (MapB), and *M. sinensis* ‘November Sunset’ (MapC), are typically winter hardy in Urbana. Nearly all MapA progeny plants survived and grew vigorously in spring 2014, whereas in MapB and MapC, many progeny plants did not survive the winter, and most of the survivors were severely damaged, with poor vigor. Negative correlations between overwintering ability and spring regrowth date and autumn dormancy date suggested that the genotypes most likely to survive winters were those that emerged early in spring and/or went dormant early in autumn. Using joint-population analysis, we identified 53 quantitative trait loci (QTLs) for nine adaptation traits, including nine QTLs for overwintering ability and 11 for spring hardiness scores. Many biologically intuitive candidate genes were observed within or near the QTLs detected in this study, suggesting their validity and potential for further study.

**KEYWORDS**
adaptation, hardiness, interconnected populations, *Miscanthus*, overwintering ability, QTL mapping
1 | INTRODUCTION

*Miscanthus × giganteus* (M×g), which is an interspecific hybrid of *M. sacchariflorus* and *M. sinensis*, is the primary species of *Miscanthus* for biomass production (Hodkinson & Renvoize, 2001; Linde-Laursen, 1993). Currently, one sterile triploid clone of M×g, ‘1993-1780’ (syn. ‘Illinois’), accounts for nearly all *Miscanthus* biomass production in North America and Europe (Clifton-Brown, Chiang, & Hodkinson, 2008; Clifton-Brown & Lewandowski, 2002; Clifton-Brown, Stampfl, & Jones, 2004; Głowacka et al., 2015; Heaton, Dohleman, & Long, 2008; Heaton, Voigt, & Long, 2004; Somerville, Youngs, Taylor, Davis, & Long, 2010). Although M×g ‘1993-1780’ is high-yielding, insufficient winter hardiness can cause severe plant losses in cold temperate environments, especially during the first winter after planting, and losses in yield in mature stands (Burner, Tew, Harvey, & Belesky, 2009; Christian & Haase, 2001; Clifton-Brown & Lewandowski, 2000; Clifton-Brown et al., 2001; Dong, Green, et al., 2018; Maughan et al., 2011). Thus, in regions where average annual minimum temperatures are −26.1°C (USDA hardiness zone 5b; U.S. Department of Agriculture, 2012) or lower, inconsistent stand establishment and productivity of M×g ‘1993-1780’ results in great economic risk that is unacceptable for commercial agricultural production. Because M×g ‘1993-1780’ is a sterile triploid, it cannot be readily used as a parent to genetically improve *Miscanthus*. However, an alternative approach to improve overwintering ability in *Miscanthus* would be to breed within M×g’s fertile parental species, *M. sacchariflorus* and *M. sinensis*, and then make the interspecific cross to generate new M×g genotypes from which to select improved cultivars (Sacks, Juvik, Lin, Stewart, & Yamada, 2013).

Genetic mapping to identify quantitative trait loci (QTLs) associated with valuable traits is a first critical step to enable marker-assisted selection, which can be especially valuable for breeding perennial crops like *Miscanthus* that have multi-year selection cycles. As *Miscanthus* is an obligately outcrossing species due to self-incompatibility, genetic mapping has primarily been conducted using the pseudo-testcross strategy on F₁ progeny (Atienza, Satovic, Petersen, Dolstra, & Martín, 2002; Dong, Liu, et al., 2018; Ge et al., 2018; Gifford, Chae, Swaminathan, Moose, & Juvik, 2015; Grattapaglia & Sederoff, 1994; Kim et al., 2012; Liu et al., 2016; Ma et al., 2012; Swaminathan et al., 2012; van der Weijde et al., 2017). However, few QTL studies on *Miscanthus* have been published and these have focused primarily on yield traits of *M. sinensis*; to the best of our knowledge, none have been published on overwintering ability.

In this study, year-3 plants of three interconnected *Miscanthus* F₁ populations that shared a common parent were evaluated in Urbana, IL for their overwintering ability and adaptation traits after the especially cold winter of 2013–2014 (minimum soil temperature at 10 cm of −6.2°C on 29 January 2014 and a minimum air temperature of −25.3°C on 6 January 2014). The objectives of this study were to (a) quantify phenotypic and genotypic variation for overwintering ability and adaptation traits of year-3 mature plants in these three *Miscanthus* populations; and (b) identify QTLs and dissect the allelic effects.

2 | MATERIALS AND METHODS

2.1 | Plant materials and experimental design

Three interconnected diploid F₁ populations that we previously studied for biomass traits (Dong, Liu, et al., 2018) were evaluated for overwintering ability of established year-3 plants over the winter of 2013–2014 in Urbana, IL (Table 1). Because *Miscanthus* is self-incompatible, individuals are highly heterozygous and F₁ populations segregate. Briefly, the three populations were developed using *M. sinensis* ssp. *condensatus* ‘Cosmopolitan’

| Table 1 | Three interconnected diploid F₁ *Miscanthus* populations (A–C) evaluated in this study. Note that the parent ‘Cosmopolitan’ is common to all three full-sib families |
|---------|-------------------------------------------------------------|
| Population | N | Parent | Parent characteristics |
| MapA | 281 | ♀ *M. sacchariflorus* ‘Robustus’ | Early flowering, thin stem, rhizomatous, cold hardy |
| | | ♂ *M. sinensis* ‘Cosmopolitan’ | Late flowering, thick stem, tall plant, cold sensitive |
| MapB | 243 | ♀ *M. sinensis* ‘Cosmopolitan’ | Late flowering, thick stem, tall plant, cold sensitive |
| | | ♂ *M. sinensis* ‘Silberturm’ | Late flowering, thick stem, cold hardy |
| MapC | 128 | ♀ *M. sinensis* ‘November Sunset’ | Late flowering, thick stem, cold hardy |
| | | ♂ *M. sinensis* ‘Cosmopolitan’ | Late flowering, thick stem, tall plant, cold sensitive |

Note. All parents are ornamental cultivars.
as an in-common parent, and the three other parents were *M. sacchariflorus* ‘Robustus’ (MapA), *M. sinensis* ‘Silberturm’ (MapB), and *M. sinensis* ‘November Sunset’ (MapC). All of the parents are diploid (Table 1). Three field trials, one for each population, were planted in close proximity to each other at the University of Illinois Energy Farm in Urbana, IL (40°3′N, 88°11′W) in June 2011. Each field trial was a randomized complete block design with three replications. Each plot contained one plant. Spacing between and within rows was 1.5 m. In each of the field trials, the same set of 13 control genotypes, including the parents of each population, were planted; thus, the trials were analogous to locations for the control set of genotypes (but all on the same farm in close proximity to each other), and this enabled testing of genotype by trial interactions. Detailed information on population development, field trials, and management was described previously in Dong, Liu, et al. (2018).

2.2 | Data collection

Data for nine adaptation traits (Table 2) were collected on the three populations from 2013 (Year-3) to 2014 (Year-4). In 2013, we collected data on spring regrowth date, which was the date of tiller emergence; autumn dormancy date, which was the date of yellowing or nongreen leaves on ≥25% of the plant; number of growing days, which was calculated by subtracting autumn dormancy date by spring regrowth date; and autumn frost damage score, which was used to estimate percentage of total leaf area that was damaged based on 0–11 scale, where 0 = no frost damage and 11 = complete frost damage. In the spring of 2014, we phenotyped hardness score and vigor of live plants in both May and June. Hardiness score was recorded as the ratio of number of new tillers to the number of previous year’s tillers with an increment of 10%. Vigor score was a 1–9 scale based on the Standard Evaluation System for Rice (IRRI, 2002), where 1 = extremely vigorous, 3 = above average vigor, 5 = average vigor, 7 = below average vigor, and 9 = weak. Overwintering ability data were calculated based on survival data of 2013 autumn and 2014 spring as follows, 0: plant was alive in 2013 autumn but dead in 2014 spring; 1: plant was alive in both 2013 autumn and 2014 spring. Only plants that were alive in autumn 2013 were evaluated for overwintering ability. Thus, all nine traits had one year of data (Table 2).

| Trait (unit)                      | Abbreviation | Trait description and evaluation system                                                                                                                                 |
|----------------------------------|--------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Overwintering ability (Prpn)     | OWA          | Recorded if the plant was dead or alive. Data taken in late October and again during the last week of following May. Dead plant was recorded as 0, and alive plant was recorded as 1. Missing plants were recorded as NA. Overwintering ability was then calculated from survival data as follows: 0, plant was alive in previous year’s autumn but was dead in current year’s spring; 1, plant was alive in previous year’s autumn and also regrew in current year’s spring. |
| Spring regrowth date (days)      | SRD          | Date of emergence of new green tissue, either by pushing older material above the previous year’s cut height or by the emergence of new shoots. Data were recorded weekly |
| Autumn dormancy date (days)      | ADD          | Date of onset of dormancy (yellowing or nongreen leaves on ≥25% of plant). Recorded weekly                                                                                   |
| Growing days (days)              | GD           | Calculated by subtracting autumn dormancy date from the spring regrowth date                                                                                                                                                      |
| Autumn frost damage (%)          | AFD          | Estimated percentage of total leaf area that was damaged using 0–11 scale. 0: No Frost Damage; 1: 1%–10% Frost Damage; 2: 11%–20% Frost Damage; 3: 21%–30% Frost Damage; 4: 31%–40% Frost Damage; 5: 41%–50% Frost Damage; 6: 51%–60% Frost Damage; 7: 61%–70% Frost Damage; 8: 71%–80% Frost Damage; 9: 81%–90% Frost Damage; 10: 91%–99% Frost Damage; 11: Complete Frost Damage |
| May hardness (%)                 | MH           | The ratio of the number of new live tillers in the spring to the number of tillers that grew in the previous year. Recorded in 10% increment with a cap of 100%                                                                            |
| June hardness (%)                | JH           | Based on the same evaluation system for hardiness score in May but data were recorded again in June                                                                             |
| May vigor (1–9 score)            | MVg          | Visual rating on 1–9 scale. 1: Extremely vigorous; 3: Above average vigor; 5: Average vigor; 7: Below average vigor; 9: Weak                                                                                   |
| June vigor (1–9 score)           | JVg          | Based on the same rating scale for vigor score in May but data were recorded again in June                                                                                                                                     |
2.3  |  Statistical analysis of phenotypic data

Initial analyses of variance (ANOVAs) were conducted with only the 13 control entries that were planted in each trial to test for possible interactions between genotype (accession) and trial using the MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC, USA) following Equation (1):

\[ y_{ijk} = \mu + T_i + G_j + TG_{ij} + B(T)_{ik} + \epsilon_{ijk} \]  

(1)

where \( y \) is the quantitative phenotypic value, \( \mu \) is the mean, \( T \) is the trial (three adjacent field trials, one for each population), \( G \) is the genotype, \( TG \) is genotype by trial interaction, \( B(T) \) is the block nested in trial, and \( \epsilon \) is error. Trial and genotype were set as fixed, and block was set as random. Estimates of variance components and of proportion of total variance were also calculated by conducting ANOVAs where all sources were considered as random effects. For overwintering ability, which is a binary trait, the aforementioned model terms were assessed using logistic regression and their significances were determined with chi-square test. Because no significant environment by genotype by environment interactions were detected based on the mixed-model ANOVAs of the 13 control entries at \( \alpha = 0.001 \), and the genotype by trial interaction term accounted for a minimal amount of the total variance (0%–9%) in the completely random model for seven of the nine traits (Supporting information Table S1), direct comparisons among the three trials were made, for the nine traits, in a second set of ANOVAs performed following Equation (2):

\[ y_{ijk} = \mu + T_i + G_j + B(T)_{ik} + \epsilon_{ijk} \]  

(2)

Trial and genotype were set as fixed, and block was set as random. Subsequent ANOVAs were also conducted independently for each of the nine traits within each trial following Equation (3):

\[ y_{ijk} = \mu + G_j + B_{ij} + \epsilon_{ij} \]  

(3)

where genotype was treated as fixed and block was treated as random, and least squares means (LS means) were calculated. Broad-sense heritability \( (H^2) \) was estimated using the Equation (3) as described above except that genotype was set as random:

\[ H^2 = \frac{\sigma^2_g}{\left(\frac{\sigma^2_g + \sigma^2_e}{B}\right)} \]  

(4)

where \( \sigma^2_g \) is the genetic variance, \( \sigma^2_e \) is the error variance, and \( B \) is the number of blocks. Pearson’s correlation coefficients among the nine traits were calculated using the CORR procedure in SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Genetic correlation coefficients were calculated with SAS procedure GLM using the MANOVA option using the following Equation (5):

\[ r_g = \frac{\sigma_{c(xy)}}{\left(\frac{\sigma^2_e \sigma^2_g}{\sigma^2_{c(xy)}}\right)^{1/2}} \]  

(5)

where \( \sigma^2_{c(xy)} \) and \( \sigma^2_{e(xy)} \) are the genetic variances for traits \( x \) and \( y \), and \( \sigma^2_{c(xy)} \) is the corresponding covariance between two traits.

2.4  |  QTL analyses

The Miscanthus consensus genetic map constructed in Dong, Liu, et al. (2018) was used for QTL analyses in TASSEL 5 (Bradbury et al., 2007). For each of the three interconnected \( F_1 \) populations, single-population analyses were performed by fitting a linkage model using stepwise regression of trait LS means on markers. Each marker entered or exited the model based on empirical \( \alpha = 0.05 \) threshold determined by a permutation procedure described in Churchill and Doerge (1994) that was conducted 1,000 times. Joint linkage analyses across all three populations were performed following a similar strategy, except that first a population main effect was fit in the joint stepwise regression model followed by the selection of marker effects nested within population to enter or exit the model based on the \( p \)-value calculated for the \( F \)-test (Bradbury et al., 2007; Buckler et al., 2009). An appropriate \( p \)-value cutoff was also determined based on 1,000 permutation test at \( \alpha = 0.05 \).

3  |  RESULTS

Significant differences among the 13 control genotypes (which included the parents of the three populations; Supporting information Tables S1 and S2) were observed for all of the nine traits. After the especially cold winter of 2013–2014 at Urbana, IL (minimum air temperature of \(-25.3°C\); minimum soil temperature at 10 cm of \(-6.2°C\)), most of the 13 control entries, which were mainly ornamental *M. sinensis* cultivars, were damaged and/or dead, even though they were large established plants that had grown well for three seasons prior (Supporting information Table S2, Figures 1 and 2). However, all plants of the commercial biomass cultivar M×g ‘1993-1780’ survived the winter. Although May hardiness and vigor scores for M×g ‘1993-1780’ indicated that the plants were winter damaged, June hardiness and vigor scores indicated that these mature plants fully recovered by the end of spring 2014 (Figure 2, Supporting information Table S2). Among the three interconnected \( F_1 \) populations, large and highly significant differences were observed in the nine traits except for the 2013 growing days (Supporting information Table S3, Figures 1 and 2).

Notably, nearly all MapA progeny plants survived with high hardiness scores (mean May hardiness of 90%; Table 3) and grew vigorously (low vigor scores; mean May vigor of 3.2) in spring 2014, whereas in MapB and MapC, many progeny plants did not survive the winter (mean overwintering of 0.8 for MapB, and 0.7 for MapC), and most of those that did survive were severely damaged, with low hardiness scores (mean...
May hardiness of 7 for MapB and 5 for MapC) and poor vigor (high vigor scores; mean May vigor of 8.5 for MapB and 8.8 for MapC) (Figures 1 and 2, Table 3). The differences in overwintering ability between MapA, the interspecific population, and the two M. sinensis intraspecific populations, MapB and MapC, mirrored the differences observed among their parents. In particular, all plots of M. sacchariflorus ‘Robustus’, the unique parent of MapA, survived the winter with high hardiness scores and strong vigor (Table 3, Figures 1 and 2). Although MapB’s unique parent, M. sinensis ‘Silverturm’, survived the winter with high frequency, its hardiness and vigor were poor (Table 3, Figures 1 and 2). The MapC unique parent, M. sinensis ‘November Sunset’, had only 70% of plots survive the winter and the survivors had poor winter hardiness and vigor (Table 3, Figures 1 and 2). In contrast to the lower average winter hardiness of the MapC progeny and the MapC unique parents, the MapC progeny had substantially less frost damage in autumn 2013 than the other two populations (Figure 2, Table 3). The parent in common to all three populations, M. sinensis ssp. condensatus ‘Cosmopolitan’, was the least hardy of all the parents, with fewer than half the plots surviving the winter, and those that did survive had poor hardiness scores and vigor (Table 3, Figures 1 and 2). Within each of the three full-sib families, significant differences were observed among genotypes (i.e. progenies) for all traits except MapA overwintering ability, MapB May vigor, and MapC autumn frost damage, May hardiness, May and June vigor, and overwintering ability (Supporting information Table S4). Large differences were observed within the three populations for number of growing days in the 2013 season, prior to the strong winter of 2013–2014 (Figure 2, Table 3). MapA progeny, like their M. sacchariflorus ‘Robustus’ parent, typically grew for substantially fewer days (mean of 183 days for the progeny and 163 days for the parent; Table 3) than MapB and MapC progeny (mean of 199 and 199 days, respectively; Table 3) and their unique parents (mean of 188 and 202 days, respectively; Table 3). The number of growing days ranged from 159 to 209 days in MapA, and 177 to 209 days in MapB and MapC (Table 3). Given that the three populations emerged at a similar time in the spring of 2013 (average spring regrowth date was April 20, April 21 and April 23 in MapA, MapB and MapC, respectively; Figure 2, Table 3), the variation in number of growing days was primarily due to differences in autumn dormancy date. However, spring regrowth dates among progeny were more uniform in MapA (April 19–April 30) than in MapB and MapC (April 19 to May 21 and April 19 to May 19, respectively). In contrast, for autumn dormancy date, MapA had a wide range, from September 30 to November 15, whereas individuals in MapB and MapC went dormant between October 28 and November 15. Broad-sense heritability estimates were typically low to moderate for MapB and MapC (0.17–0.59; Table 3). However, for MapA, high broad-sense heritabilities were obtained for autumn dormancy (0.82), growing days (0.81), May and June hardiness (0.73 and 0.69, respectively), and May and June vigor (0.78 and 0.83, respectively). Because nearly all the plants survived in MapA (avg. overwintering ability: 1.0, Table 3), heritability for overwintering ability could not be estimated and thus was calculated only for the other two populations (0.37 in MapB, 0.35 in MapC). All three populations had similar heritabilities for spring regrowth date (0.31–0.45), and moderate to high heritabilities for autumn dormancy date (0.43–0.82) and growing days (0.41–0.81). For autumn frost damage, heritabilities were 0.70, 0.59 and 0.17 for MapA, MapB, and MapC, respectively. MapB and MapC had low heritabilities for hardiness and vigor in May (0.17–0.21) due to limited variation, but had higher heritabilities in June (0.32–0.41) as differences among genotypes in ability to recover were observed.

Many significant genetic correlations existed between traits in these three interconnected populations (Table 4). Overwintering ability showed moderate-to-high negative genetic correlations with spring regrowth date (−0.79 in MapB, −0.59 in MapC) and with autumn dormancy date (−0.82 in MapB; −0.47 in MapC), suggesting that plants that emerged earlier in spring or went dormant earlier in autumn were more likely to survive the cold winter. Between overwintering ability and hardiness scores, low-to-moderate positive genetic correlations were observed (0.31–0.50 in MapB; 0.40–0.55 in MapC). Moreover, significant and negative genetic correlations existed between hardiness scores and vigor scores (−0.38 to −0.93) across the three populations (Table 4), and this conformed to our expectations because both higher hardiness score and lower vigor score were two indicators of greater cold tolerance. Phenotypic correlations and genetic correlations showed similar relationships between traits (Table 4). Single-population analyses identified 26, 10, and 3 QTLs among nine traits in MapA, MapB, and MapC, respectively (Figure 3, Supporting information Figure S1 and Table S5). Each of the QTLs identified was unique to the population in which they were found. Thus, single-population analyses detected a total of 39 QTLs. In contrast, joint-population analyses identified 53 QTLs among the nine traits (Figure 3 and Table 5, Supporting information Figure S1). There were 31 QTLs in common between the single-population analyses and the joint-population analyses (Figure 3 and Table 5, Supporting information Figure S1 and Table S5). For overwintering ability, no QTL were detected from MapA because nearly all plants in this population survived (Figure 3, Supporting information Table S5), whereas one QTL was identified each in MapB and MapC (LG 9 in MapB; LG 1 in MapC; Figure 3, Supporting information Table S5). In contrast, joint-population analyses identified nine QTLs for overwintering ability across the three populations, including the two QTLs detected in MapB and MapC (Figure 3, Table 5). Similarly, joint-population analyses identified four QTLs for May hardiness and seven for June.
hardiness (Table 5). Among the nine traits, the greatest number of QTL detected was for autumn dormancy date (14 in the joint-population analysis), which corresponded to the substantial phenotypic variation observed for this trait in the three populations (Figure 2). Notably, joint-population analyses produced significantly higher resolution for QTLs (i.e., smaller confidence intervals) than single-population analyses, and this was consistent with our previous findings from genetic mapping for biomass yield traits (Dong et al., 2018). Average confidence interval across the 31 in-common QTLs was 27.4 ± 1.9 cM for single-population analyses but was only 17.6 ± 1.4 cM for joint-population analyses (Figure S1). Similarly, average confidence interval across all QTLs was 28.2 ± 1.6 cM for single-population analyses but only 20.7 ± 1.1 cM for joint-population analyses. Additive effects of alleles inherited from each of the three parents unique to each population (M. sacchariflorus ‘Robustus’ in MapA, M. sinensis ‘Silberturm’ in MapB, M. sinensis ‘November Sunset’ in MapC) were estimated using allelic effects of the in-common parent, M. sinensis ‘Cosmopolitan’, as the baseline for both single-population and joint-population analyses (Supporting information Table S5, Table 5). However, joint-population analysis has been shown to be more accurate in estimating QTL allelic effects than single-population analyses (Buckler et al., 2009; Li, Bradbury, Ersoz, Buckler, & Wang, 2011), so we focused our attention on estimates from the joint-population analyses (Table 5). Consistent with our field observations that M. sacchariflorus ‘Robustus’ was strongly winter hardy and contributed this trait to its progeny (Figures 1 and 2), we found that it was the primary source of large effect alleles for increasing May hardiness and improving May and June vigor (lower values), although some large effect alleles for reduced May hardiness were also observed. Due to the uniform survival of the MapA progeny, no large effects were estimated for M. sacchariflorus ‘Robustus’ alleles at the significant loci detected for overwintering ability, although other large effect alleles must have existed at undetected loci, putatively homozygous in ‘Robustus’. The M. sinensis ‘Silberturm’ and ‘November Sunset’ parents predominantly contributed alleles that substantially reduced June hardiness and overwintering ability, but some large positive effect alleles were also observed from these parents, indicating opportunities for selection even within the ornamental M. sinensis germplasm pool. M. sacchariflorus ‘Robustus’ also contributed most of the large effect alleles for decreasing the number of growing days and the autumn dormancy date, which was consistent with our field observation that M. sacchariflorus ‘Robustus’ went dormant very early (September 30, 2013) and conferred this trait to many of its progeny.

4 | DISCUSSION

4.1 | Comparison between this study and prior studies for winter hardiness of M×g

In contrast to prior studies on winter hardiness and freeze-tolerance of Miscanthus (Clifton-Brown & Lewandowski, 2000; Fonteyne et al., 2016; Peixoto et al., 2015), this study evaluated the response of mature, established field plantings after the third growing season, to an especially cold winter (minimum temperatures for air and 10 cm below bare soil were −25.3°C and −6.2°C in 2013–2014). Although all mature plants of the commercial check, M×g ‘1993-1780’, survived the 2013–2014 winter in this study at Urbana, IL, hardiness, and vigor scores from May and June 2014 indicated that the plants were damaged but appeared to subsequently recover by late spring. In contrast to the survival of mature M×g ‘1993-1780’ plants in this study, in a parallel study of first year M×g ‘1993-1780’ plants, we observed losses of 29% during the 2013–2014 winter at Urbana, IL (Dong et al., 2018). Previous studies have estimated the temperature at which 50% of isolated rhizomes from mature plants were killed (LT50) for M×g ‘1993-1780’ to be −2.6°C (Fonteyne et al., 2016), −3.4°C (Clifton-Brown & Lewandowski, 2000), or −4.4°C (Peixoto et al., 2015), but in the field, insulation of belowground rhizomes from the plant’s aboveground crown, and avoidance of cold by rhizomes that grow deep in the soil, can increase overwintering ability of mature plants. For example, under sod, the minimum temperature during the 2013–2014 winter at Urbana was only −2.2°C at 10 cm and −1.4°C at 20 cm. In large areas of the Midwest US, models indicate that at 10 cm below bare soil, temperatures between −3.5°C and −6.0°C can be expected to occur in most years (Kucharik, VanLoocke, Lenters, & Motew, 2013), so breeding for adaptation to this degree of freezing and/or freeze avoidance would be a prudent breeding objective for this and similar regions.
FIGURE 2 Frequency distributions (histograms) for nine adaptation traits (trait bins on x-axis, counts on y-axis) in three interconnected diploid F1 Miscanthus populations (MapA: M. sacchariflorus (Msa) ‘Robustus’ × M. sinensis (Msi) ‘Cosmopolitan’ (n = 281); MapB: M. sinensis ‘Cosmopolitan’ × M. sinensis ‘Silberturm’ (n = 243); MapC: M. sinensis ‘November Sunset’ × M. sinensis ‘Cosmopolitan’ (n = 128)), their parents, and the high-yielding control, M. × giganteus (M×g) ‘1993-1780’, established in a field trial with three clonal replications at Urbana, IL in 2011. Four traits, including spring regrowth date, autumn dormancy date, growing days, and autumn frost damage were phenotyped in 2013. The remaining five traits, May and June plant hardiness scores (ratio of number of new tillers in spring of 2014 relative to number of tillers at the end of the 2013 growing season, expressed as a percentage), plant vigor in both May and June, and overwintering ability (proportion survival), were phenotyped in 2014 and were measures of winter hardiness of established year-3 plants. The winter of 2013–2014 was especially cold in Urbana, IL, with a minimum soil temperature at 10 cm of −6.2°C on 29 January 2014 and a minimum air temperature of −25.3°C on 6 January 2014.
4.2 | Large differences among families for third year overwintering ability and hardiness were consistent with the adaptation of the parents

The in-common parent of the three interconnected F₁ populations, *M. sinensis* spp. *condensatus* ‘Cosmopolitan’, was the least winter hardy of the four parents and 13 controls in this study, as expected. *M. sinensis* spp. *condensatus* is indigenous to maritime southern Japan (hardiness zones 9b to 10a; average annual minimum air temperature of −3.9 to 1.7°C). Thus, ‘Cosmopolitan’ is typically damaged or killed during winters in Urbana, IL (zone 5b; average annual minimum air temperature of −26.1 to −23.3°C). In contrast, the other three parents of the F₁ populations have typically performed well in central Illinois, especially...
TABLE 4  Correlation coefficients between pairs of nine adaptation traits (genetic correlations are above the diagonal and Pearson’s correlations are below the diagonal) for three interconnected diploid F$_1$ Miscanthus populations (MapA: *M. sacchariflorus* ‘Robustus’ × *M. sinensis* ‘Cosmopolitan’ (n = 281); MapB: *M. sinensis* ‘Cosmopolitan’ × *M. sinensis* ‘Silberturm’ (n = 243); MapC: *M. sinensis* ‘November Sunset’ × *M. sinensis* ‘Cosmopolitan’ (n = 128)), established in a field trial with three clonal replications at Urbana, IL in 2011

|                  | Spring regrowth date (2013) | Autumn dormancy date (2013) | Growing days (2013) | Autumn frost damage (2013) | May hardiness (2014) | June hardiness (2014) | May vigor (2014) | June vigor (2014) | Overwintering ability (2014)* |
|------------------|-----------------------------|-----------------------------|---------------------|--------------------------|---------------------|---------------------|------------------|------------------|-----------------------------|
| MapA             |                             |                             |                     |                          |                     |                     |                  |                  |                             |
| Spring regrowth date (2013) | 0.14                       | 0.30                        | -0.19               | -0.57                    | -0.35               | 0.66                | 0.67             |                  |                             |
| Autumn dormancy date (2013)  | 0.37                       | 0.99                        | -0.30               | -0.42                    | -0.27               | 0.40                | 0.38             |                  |                             |
| Growing days (2013)          | NS                         | 0.99                        | -0.27               | -0.38                    | -0.25               | 0.36                | 0.34             |                  |                             |
| Autumn frost damage (2013)   | -0.66                      | -0.45                       | -0.41               | 0.55                     | 0.21                | -0.68              | -0.63            |                  |                             |
| May hardiness (2014)         | -0.22                      | -0.19                       | -0.16               | 0.22                     | 0.66                | -0.90              | -0.81            |                  |                             |
| June hardiness (2014)        | -0.22                      | -0.11                       | -0.07               | 0.10                     | 0.42                | -0.75              | -0.82            |                  |                             |
| May vigor (2014)             | 0.23                       | 0.21                        | 0.17                | -0.30                    | -0.76               | -0.30              | 0.74             |                  |                             |
| June vigor (2014)            | 0.36                       | 0.23                        | 0.18                | -0.33                    | -0.64               | -0.55              | 0.58             |                  |                             |
| Overwintering ability (2014) |                            |                             |                     |                          |                     |                     |                  |                  |                             |
| MapB             |                             |                             |                     |                          |                     |                     |                  |                  |                             |
| Spring regrowth date (2013) | 0.13                       | -0.20                       | -0.08               | -0.93                    | -0.60               | 0.19                | 0.52             | -0.79            |                             |
| Autumn dormancy date (2013)  | 0.51                       | 0.91                        | -0.09               | -0.53                    | -0.66               | -0.05              | 0.40             | -0.82            |                             |
| Growing days (2013)          | -0.21                      | 0.81                        | NS                  | -0.16                    | -0.47               | -0.19              | 0.22             | -0.38            |                             |
| Autumn frost damage (2013)   | 0.09                       | -0.03                       | -0.08               | 0.66                     | 0.33                | -0.72              | -0.70            | 0.13             |                             |
| May hardiness (2014)         | NS                         | NS                          | NS                  | NS                       | 0.98                | -0.53              | -0.89            | 0.31             |                             |
| June hardiness (2014)        | -0.08                      | -0.14                       | -0.08               | NS                       | 0.68                | -0.81              | -0.93            | 0.50             |                             |
| May vigor (2014)             | NS                         | -0.25                       | -0.20               | 0.17                     | -0.82               | -0.52              | 0.78             |                  |                             |
| June vigor (2014)            | NS                         | NS                          | NS                  | NS                       | -0.74               | -0.86              | 0.72             |                  |                             |
| Overwintering ability (2014) | -0.18                      | -0.18                       | NS                  | NS                       | 0.85                | 0.92               |                  |                  |                             |
| MapC             |                             |                             |                     |                          |                     |                     |                  |                  |                             |
| Spring regrowth date (2013) | 0.34                       | -0.14                       | NS                  | -0.57                    | -0.29               | 0.70                | -0.17            | -0.59            |                             |
| Autumn dormancy date (2013)  | 0.46                       | 0.24                        | -0.13               | -0.26                    | -0.26               | -0.01              | -0.47            |                  |                             |
| Growing days (2013)          | NS                         | 0.4                         | NS                  | 0.60                     | 0.48                | -0.14              | -0.44            | 0.67             |                             |
| Autumn frost damage (2013)   | -0.14                      | -0.18                       | NS                  | 0.15                     | 0.95                | -0.83              | -0.77            | 0.40             |                             |

(Continues)
M. sacchariflorus ‘Robustus’, which is highly rhizomatous and cold hardy (Kaiser & Sacks, 2015). M. sacchariflorus ‘Robustus’, the unique parent of MapA, originated from northern China or eastern Russia (Clark et al., 2018) and is thus adapted to hardiness zone 3 (average annual minimum air temperature of −40.0 to −34.4°C). Consistent with its origin, M. sacchariflorus ‘Robustus’ was the most winter hardy entry among the parents and controls in the current study, with all plants surviving without any damage (Figures 1 and 2). The observed complete survival of all MapA progeny, with little or no winter-damage, indicates that ‘Robustus’ alleles for winter hardiness were dominant, and/or additively exceeded a threshold needed to survive the 2013–2014 winter in Urbana. Moreover, the results from MapA demonstrate the potential to use subtropical-adapted materials (in this case M. sinensis spp. condensatus ‘Cosmopolitan’) in Miscanthus breeding programs targeting cold temperate environments by combining the unadapted germplasm with a parent that has greater winter hardiness than is required for the target environment (in this case M. sacchariflorus ‘Robustus’). This is a valuable lesson because subtropical and tropical M. sinensis populations typically have greater biomass potential than northern populations (Lindsay Clark, unpublished data).

The relatively poor overwintering ability of MapB and MapC progeny, their parents (M. sinensis ‘Silberturm’ and M. sinensis ‘November Sunset’, respectively), and most of the controls, demonstrate a key limitation to relying entirely on the currently available ornamental Miscanthus cultivars for breeding new cultivars adapted to hardiness zones 5 and colder. However, there exist natural populations of M. sinensis from northern Japan, northern China, and eastern Russia that are adapted to colder winters than the ornamental M. sinensis cultivars currently available in the United States and Europe (Clark et al., 2014, 2016, 2015; Lim et al., 2014; Yan et al., 2012). Although the winter of 2013–2014 in Urbana was near the lower end of the range expected for minimum annual air temperatures in central Illinois based on a 30-year record (1976–2005), the results of this study highlight the importance of breeding for adaptation to the coldest winter expected for a target environment, especially given that Miscanthus production fields are expensive to establish and growers may plan to keep a planting for ten or more years. We have previously shown that the ornamental M. sinensis cultivars available in the United States and Europe were derived from wild populations of central and southern Japan (hardiness zones 8a to 10a; average annual minimum air temperature of −12.2 to 1.7°C; Clark et al., 2014, 2015), although about half the ornamental cultivars sold as M. sinensis in fact have some M. sacchariflorus ancestry, presumably the result of past efforts by ornamental grass breeders in Germany to increase winter hardiness.
and obtain earlier flowering (Clark et al., 2014). Breeders of *Miscanthus* for biomass production in cold climates may use a similar introgression strategy as was used for prior ornamental cultivar breeding, but must also select for greater biomass yield, in addition to temperate-climate adaptation.
FIGURE 3 Chromosomal locations of QTLs identified in each of three single-population analyses and one joint-population (JP) analysis. Phenotypic data were from three diploid F1 Miscanthus populations (MapA: M. sacchariflorus (Msa) ‘Robustus’ × M. sinensis (Msi) ‘Cosmopolitan’ (n = 281); MapB: M. sinensis ‘Cosmopolitan’ × M. sinensis ‘Silberturm’ (n = 243); MapC: M. sinensis ‘November Sunset’ × M. sinensis ‘Cosmopolitan’ (n = 128)) established in a field trial with three clonal replications at Urbana, IL in 2011. Nine traits were included in QTL analyses. Four traits, including spring regrowth date, autumn dormancy date, growing days, and autumn frost damage were phenotyped in 2013. The remaining five traits, May and June plant hardiness scores (ratio of number of new tillers in spring of 2014 relative to number of tillers at the end of the 2013 growing season, expressed as a percentage), plant vigor in both May and June, and overwintering ability (proportion survival), were phenotyped in 2014 and were measures of winter hardness of established year-3 plants. The winter of 2013–2014 was especially cold in Urbana, IL, with a minimum soil temperature at 10 cm of −6.2°C on 29 January 2014 and a minimum air temperature of −25.3°C on 6 January 2014. Horizontal axis represents marker position on the Miscanthus consensus genetic map. Colored bars represent confidence intervals of QTLs; filled circles and stars show peak positions of QTLs identified for traits phenotyped in 2013 (filled circles) and 2014 (stars), respectively.

4.3 Potential for indirect selection of overwintering ability and hardiness based on spring regrowth date and autumn dormancy date

Moderate-to-strong genetic correlations between early spring regrowth in 2013 and greater overwintering ability during the subsequent winter for both MapB and MapC, and with greater May hardness in all three populations suggests an opportunity for indirect selection for increased winter hardness even in years that do not have especially cold winters by selecting for early spring regrowth. In perennial ryegrass (Lolium perenne L.), Yu et al. (2015) similarly observed that cultivars that emerged earliest in the spring were most likely to survive the next winter. We also observed that early autumn dormancy was genetically correlated with greater overwintering ability and May hardness, although this was less consistent among the populations relative to early spring regrowth date. For MapC, which had a substantially later mean autumn dormancy date than MapB (Table 3), there was a weaker association between early dormancy and overwintering ability than for MapB (Table 4), suggesting that as dormancy date became later, the opportunity to indirectly improve overwintering was less. Thus, breeding Miscanthus with improved overwintering ability might be achieved by selecting plants with early spring emergence date and/or autumn dormancy date, although this must be balanced by the potential risks of early spring or autumn freezes (Farrell, Clifton-Brown, Lewandowski, & Jones, 2006; Kaiser & Sacks, 2015) and the desire to maximize biomass yield-potential by maximizing photosynthesis per season.

4.4 QTL mapping reveals a mixture of allelic effects from each parent

Overall, QTL mapping of the three interconnected bi-parental Miscanthus populations was more effective with joint-population analysis (more QTL identified and smaller confidence intervals) than single-population analysis (Supporting information Table S5, Table 5 and Figure 3). With joint-population analysis, 53 QTL were detected (Figure 3), whereas only 39 were detected with single-population analyses; moreover, most of the QTL detected in single-population analyses were also detected in the joint-population analysis (31 in-common QTL). Previous studies of two to ten interconnected populations have also found that joint-population analyses identified most of the QTL identified by single-population analyses, although each method also identified some QTLs uniquely (Chandler et al., 2013; Dong, Liu, et al., 2018; Li et al., 2011; Yang et al., 2013). Joint linkage analysis is expected to increase the power to detect QTL, and to estimate locus positions and allelic effects with greater precision than single-population analyses (Blanc, Charcosset, Mangnin, Gallalis, & Moreau, 2006; Dong, Liu, et al., 2018; Li et al., 2011; Negeri, Coles, Holland, & Balint-Kurti, 2011; Walling et al., 2000; Yang et al., 2013). Thus, joint-population analysis was expected to detect QTLs that are not identified by individual population analyses. Consistent with these expectations, in maize NAM populations, joint linkage analysis across all 25 nested RIL populations identified twice as many QTLs as analyses of single RIL populations (Buckler et al., 2009). Similarly, in the current Miscanthus study, nine QTLs were detected for overwintering ability with the joint-population analysis, whereas only two QTLs were detected for overwintering ability with the individual population analyses. Thus, joint linkage analysis can be a useful strategy for identifying QTL with greater efficiency than is possible with individual population analyses.

Relative to the in-common parent, M. sinensis spp. condensatus ‘Cosmopolitan’, a mixture of positive and negative QTL allelic effects from each of the three other parents in the interconnected population were observed for most of the traits studied (Supporting information Table S5, Table 5), indicating that there was substantial opportunity for selection in either direction. Although uniform survival of the MapA progeny prevented identification of the clearly advantageous alleles from the M. sacchariflorus ‘Robustus’ parent for this trait in the F1 generation, it should be possible to detect these loci and estimate allelic effects in the F2 and/or subsequent generations. Notably, even within the relatively nonhardy parents, M. sinensis ‘Silberturm’ and M. sinensis ‘November Sunset’, alleles for substantially improved overwintering ability and May hardness were detected. Thus, opportunities exist to improve winter hardness and overwintering ability even within the relatively...
| QTL id\(^a\) | Year | QTL Peak Marker | LG | LOD | Position (cM) | PVE (%) | Left Bound (cM) | Right Bound (cM) | Allelic Effect of Msa. ‘Robustus’ |Allelic Effect of Msi. ‘Silberturm’| Allelic Effect of Msi. ‘November Sunset’ | 31 in-common QTLs\(^b\) |
|----------------|-------|-----------------|----|-----|--------------|---------|----------------|--------------------|-------------------------------------|----------------------------------|-----------------------------------|---------------------|
| SRD1           | 2013  | UIMiscanthus000464 | 4  | 8.3 | 34.3         | 7.3     | 28.7           | 39.9               | −0.91                              | 1.11                             | −21.86                           |                     |
| SRD2           | 2013  | UIMiscanthus082668 | 10 | 5.2 | 31.0         | 5.3     | 23.3           | 38.6               | −6.60                              | 1.03                             | −0.70                             | Y                   |
| ADD1           | 2013  | UIMiscanthus086572 | 1  | 6.5 | 120.9        | 3.6     | 109.7          | 132.0              | −11.41                             | 2.25                             | 0.62                              |                     |
| ADD2           | 2013  | UIMiscanthus008730 | 3  | 3.9 | 72.4         | 2.2     | 53.5           | 91.2               | −8.58                              | −1.53                            | 1.40                              |                     |
| ADD3           | 2013  | UIMiscanthus061128 | 4  | 5.4 | 69.2         | 3.0     | 55.6           | 82.7               | −8.82                              | −5.87                            | −2.29                             |                     |
| ADD4           | 2013  | UIMiscanthus019698 | 6  | 3.7 | 67.4         | 2.1     | 47.7           | 87.1               | 8.20                               | 1.58                             | 3.17                              | Y                   |
| ADD5           | 2013  | UIMiscanthus003691 | 7  | 11.8| 65.4         | 6.8     | 59.5           | 71.4               | 15.75                              | −0.45                            | 0.36                              | Y                   |
| ADD6           | 2013  | UIMiscanthus074683 | 8  | 4.2 | 30.9         | 2.3     | 13.4           | 48.4               | −11.14                             | −4.19                            | −4.49                             |                     |
| ADD7           | 2013  | UIMiscanthus088390 | 9  | 3.5 | 80.4         | 2.0     | 59.8           | 100.9              | 6.79                               | 5.83                             | −0.34                             |                     |
| ADD8           | 2013  | UIMiscanthus082668 | 10 | 12.6| 31.0         | 7.3     | 25.4           | 36.5               | −16.65                             | 2.36                             | 0.56                              | Y                   |
| ADD9           | 2013  | UIMiscanthus084311 | 12 | 6.5 | 81.2         | 3.7     | 70.1           | 88.2               | 11.38                              | 0.70                             | 1.87                              | Y                   |
| ADD10          | 2013  | UIMiscanthus002312 | 13 | 4.4 | 51.3         | 2.4     | 34.6           | 68.0               | −12.06                             | 1.54                             | 0.78                              | Y                   |
| ADD11          | 2013  | UIMiscanthus001120 | 14 | 5.8 | 47.6         | 3.2     | 35.1           | 60.2               | 14.69                              | 5.68                             | 0.84                              | Y                   |
| ADD12          | 2013  | UIMiscanthus086532 | 17 | 5.7 | 69.4         | 3.2     | 56.7           | 82.0               | 6.56                               | 2.05                             | −0.37                             | Y                   |
| ADD13          | 2013  | UIMiscanthus071035 | 18 | 6.2 | 26.5         | 3.5     | 14.7           | 38.2               | 14.58                              | −3.77                            | −1.79                             | Y                   |
| ADD14          | 2013  | UIMiscanthus040489 | 19 | 8.3 | 96.2         | 4.7     | 87.6           | 98.9               | −13.04                             | 0.72                             | −0.26                             | Y                   |
| GD1            | 2013  | UIMiscanthus020369 | 1  | 4.4 | 117.9        | 3.1     | 104.8          | 130.9              | −10.43                             | 3.46                             | 2.16                              |                     |
| GD2            | 2013  | UIMiscanthus003691 | 7  | 9.1 | 65.4         | 6.5     | 59.2           | 71.7               | 5.56                               | 1.00                             | 2.50                              | Y                   |
| GD3            | 2013  | UIMiscanthus082668 | 10 | 5.5 | 31.0         | 3.9     | 20.7           | 41.3               | −12.75                             | −0.05                            | 0.19                              | Y                   |
| GD4            | 2013  | UIMiscanthus084311 | 12 | 5.0 | 81.2         | 3.6     | 69.7           | 88.2               | 11.66                              | 0.14                             | −0.81                             | Y                   |
| GD5            | 2013  | UIMiscanthus002312 | 13 | 5.2 | 51.3         | 3.7     | 40.3           | 62.3               | −15.32                             | 1.08                             | 1.33                              | Y                   |
| GD6            | 2013  | UIMiscanthus086532 | 17 | 5.9 | 69.4         | 4.2     | 59.7           | 79.0               | 9.79                               | 3.52                             | −5.21                             | Y                   |
| GD7            | 2013  | UIMiscanthus074528 | 18 | 5.1 | 26.7         | 3.6     | 15.5           | 37.9               | 10.86                              | −5.28                            | −1.86                             | Y                   |
| GD8            | 2013  | UIMiscanthus040489 | 19 | 6.1 | 96.2         | 4.3     | 86.8           | 98.9               | −12.74                             | 0.89                             | −1.46                             | Y                   |
| AFD1           | 2013  | UIMiscanthus072864 | 3  | 3.9 | 24.9         | 3.1     | 11.9           | 37.8               | 0.62                               | 1.24                             | 0.23                              |                     |
| AFD2           | 2013  | UIMiscanthus082668 | 10 | 17.1| 31.0         | 14.5    | 28.2           | 33.8               | 2.88                               | 0.21                             | −0.01                             | Y                   |
| AFD3           | 2013  | UIMiscanthus070347 | 14 | 4.1 | 51.1         | 3.3     | 38.9           | 63.3               | −0.71                              | −1.57                            | −0.03                             |                     |
| AFD4           | 2013  | UIMiscanthus069016 | 16 | 5.4 | 37.0         | 4.4     | 27.8           | 46.2               | −0.24                              | 1.72                             | −0.01                             |                     |
| MH1            | 2014  | UIMiscanthus015837 | 4  | 5.5 | 56.5         | 3.6     | 45.2           | 67.8               | −15.14                             | −0.55                            | −6.17                             | Y                   |

(Continues)
| QTL id<sup>a</sup> | Year | QTL Peak Marker | LG | LOD | Position (cM) | PVE (%) | Left Bound (cM) | Right Bound (cM) | Allelic Effect of Msa. 'Robustus' | Allelic Effect of Msi. 'Silberturm' | Allelic Effect of Msi. 'November Sunset'<sup>b</sup> | 31 in-common QTLs<sup>b</sup> |
|-----------------|------|-----------------|----|-----|--------------|--------|----------------|----------------|----------------|-------------------------------|--------------------------------------|--------------------------------------|----------------------|
| MH2             | 2014 | UIMiscanthus082668 | 10 | 22.9| 31.0         | 15.9   | 28.4           | 33.5           | 32.83                       | −8.48                          | −0.54                               | Y                    |
| MH3             | 2014 | UIMiscanthus068844 | 11 | 4.4 | 56.8         | 2.8    | 42.5           | 71.2           | 15.61                       | −4.24                          | 0.80                                |                      |
| MH4             | 2014 | UIMiscanthus056112 | 19 | 5.0 | 89.4         | 3.2    | 76.8           | 98.9           | −14.60                      | 0.68                           | 1.77                                | Y                    |
| JH1             | 2014 | UIMiscanthus000815 | 1  | 7.5 | 55.9         | 5.7    | 48.7           | 63.1           | −0.20                       | −20.87                         | 41.64                               | Y                    |
| JH2             | 2014 | UIMiscanthus002478 | 1  | 4.7 | 105.8        | 3.5    | 94.3           | 117.4          | −1.24                       | −21.97                         | −7.47                                | Y                    |
| JH3             | 2014 | UIMiscanthus001689 | 2  | 4.3 | 17.6         | 3.2    | 4.9            | 30.2           | 1.52                        | −16.56                         | 19.31                               |                      |
| JH4             | 2014 | UIMiscanthus0073786 | 3  | 4.0 | 65.9         | 2.9    | 52.1           | 79.7           | 0.29                        | 12.61                          | −26.98                              |                      |
| JH5             | 2014 | UIMiscanthus090014 | 4  | 8.1 | 89.8         | 6.1    | 83.1           | 96.4           | 1.25                        | 29.56                          | 0.08                                | Y                    |
| JH6             | 2014 | UIMiscanthus084022 | 5  | 4.1 | 12.2         | 3.1    | 0.0            | 25.5           | 0.66                        | −20.64                         | −6.52                               |                      |
| JH7             | 2014 | UIMiscanthus0088409 | 6  | 7.1 | 86.0         | 5.3    | 78.4           | 93.6           | −2.19                       | −25.04                         | −8.78                               | Y                    |
| Mvg1            | 2014 | UIMiscanthus081890 | 4  | 5.7 | 52.3         | 4.1    | 42.4           | 62.2           | 1.52                        | −0.05                          | −0.14                               | Y                    |
| Mvg2            | 2014 | UIMiscanthus082668 | 10 | 39.0| 31.0         | 31.7   | 29.7           | 32.2           | −4.34                       | 0.34                           | 0.38                                | Y                    |
| Mvg3            | 2014 | UIMiscanthus089742 | 11 | 7.1 | 52.3         | 5.1    | 44.3           | 60.2           | −1.94                       | 0.09                           | 0.24                                | Y                    |
| Mvg4            | 2014 | UIMiscanthus067530 | 13 | 5.1 | 43.9         | 3.7    | 32.9           | 54.9           | 1.53                        | −0.36                          | 0.25                                | Y                    |
| JVg5            | 2014 | UIMiscanthus082668 | 10 | 8.0 | 31.0         | 6.6    | 24.8           | 37.1           | −2.13                       | 0.53                           | −0.42                               | Y                    |
| OWA1            | 2014 | UIMiscanthus061802 | 1  | 8.8 | 105.8        | 6.2    | 99.3           | 112.4          | 0.00                        | −0.15                          | −0.49                               | Y                    |
| OWA2            | 2014 | UIMiscanthus059172 | 4  | 6.1 | 72.3         | 4.3    | 62.8           | 81.8           | −0.01                       | 0.28                           | −0.26                               |                      |
| OWA3            | 2014 | UIMiscanthus069638 | 6  | 5.6 | 64.7         | 3.9    | 54.2           | 75.1           | −0.01                       | −0.32                          | 0.17                                |                      |
| OWA4            | 2014 | UIMiscanthus064552 | 8  | 5.7 | 66.1         | 4.0    | 55.9           | 76.4           | 0.00                        | 0.29                           | −1.32                               |                      |
| OWA5            | 2014 | UIMiscanthus072736 | 9  | 5.1 | 54.5         | 3.5    | 43.0           | 66.0           | 0.00                        | −0.31                          | 0.02                                | Y                    |
| OWA6            | 2014 | UIMiscanthus036875 | 11 | 3.7 | 29.4         | 2.6    | 13.4           | 45.3           | −0.01                       | −0.02                          | −1.05                               |                      |
| OWA7            | 2014 | UIMiscanthus011134 | 15 | 5.4 | 0.0          | 3.8    | 0.0            | 10.7           | 0.00                        | −0.11                          | 0.84                                |                      |
| OWA8            | 2014 | UIMiscanthus016483 | 15 | 6.0 | 38.3         | 4.2    | 28.6           | 48.0           | 0.04                        | −0.22                          | −0.50                               |                      |
| OWA9            | 2014 | UIMiscanthus012370 | 18 | 4.5 | 85.2         | 3.2    | 72.3           | 98.1           | 0.00                        | −0.26                          | 0.19                                |                      |

Notes. Traits include spring regrowth date (SRD), autumn dormancy date (ADD), growing days (GD), autumn frost damage (AFD), May hardiness score (MH), June hardiness score (JH), May vigor score (Mvg), June vigor score (JVg), and overwintering ability (OWA).

<sup>a</sup>QTL id is represented by trait abbreviation followed by a number index, for example, SRD1 means spring regrowth date QTL 1. <sup>b</sup>Y indicates that QTL belongs to the 31 in-common QTLs between single-population analysis and joint-population analysis; empty cell indicate QTL is unique to joint-population analysis.
| QTL id | Year | Miscanthus SNP that has significant candidate gene hits within or near QTL CI |
|--------|------|--------------------------------------------------------------------------------|
| SRD1   | 2013 | UIMiscanthus072820 4 40.5 20.7 39.9 Sobic.002G361500 AT4G37390 GH3.2, AUR3 Auxin-responsive GH3 protein |
| ADD2   | 2013 | UIMiscanthus081054 3 64.9 53.5 91.2 Sobic.002G274800 AT4G22920 NYE1 nonyellowing 1 |
| ADD3   | 2013 | UIMiscanthus063021 4 67.9 55.6 82.7 Sobic.002G320200 AT5G36110 CYP716A1 cytochrome P450 |
| ADD4   | 2013 | UIMiscanthus075716 6 71.0 47.7 87.1 Sobic.003G272200 AT2G46510 AIB ABA-inducible BHLH-type TF |
| ADD5   | 2013 | UIMiscanthus081770 7 61.6 59.5 71.4 Sobic.007G003500 AT3G26300 CYP71B34 cytochrome P450, |
| ADD6   | 2013 | UIMiscanthus083491 8 32.6 13.4 48.4 Sobic.004G188400 AT4G08920 ATCR1 cryptochrome 1 |
| ADD9   | 2013 | UIMiscanthus085437 12 77.6 70.1 88.2 Sobic.006G245500 AT5G57390 AIL5 AINTEGUMENTA-like 5 |
| ADD12  | 2013 | UIMiscanthus061268 17 63.7 56.7 82.0 Sobic.009G183800 AT1G79320 MC6 metacaspase 6 |
| GD2    | 2013 | UIMiscanthus081770 7 61.6 59.2 71.7 Sobic.007G003500 AT3G26300 CYP71B34 cytochrome P450 |
| GD4    | 2013 | UIMiscanthus085437 12 77.6 69.7 88.2 Sobic.006G245500 AT5G57390 AIL5 AINTEGUMENTA-like 5 |
| GD6    | 2013 | UIMiscanthus061268 17 63.7 59.7 79.0 Sobic.009G183800 AT1G79320 MC6 metacaspase 6 |
| AFD4   | 2013 | UIMiscanthus002604 16 41.2 27.8 46.2 Sobic.009G183900 AT1G79320 MC6 metacaspase 6 |
| MH1    | 2014 | UIMiscanthus060389 4 59.5 45.2 67.8 Sobic.002G292300 AT2G22410 SLO1 SLOW GROWTH 1 |
| MH3    | 2014 | UIMiscanthus001639 11 49.6 42.5 71.2 Sobic.006G188900 AT1G21450 SCL1 SCARECROW-like 1 |
| JH2    | 2014 | UIMiscanthus002771 1 108.3 94.3 117.4 Sobic.001G431500 AT5G16080 CXE17 carboxylesterase 17 |
| JH4    | 2014 | UIMiscanthus081054 3 65.0 52.1 79.7 Sobic.002G274800 AT4G22920 NYE1 nonyellowing 1 |
| MVg1   | 2014 | UIMiscanthus060389 4 59.5 42.4 62.2 Sobic.002G292300 AT2G22410 SLO1 SLOW GROWTH 1 |
| MVg3   | 2014 | UIMiscanthus001639 11 49.6 44.3 60.2 Sobic.006G188900 AT1G21450 SCL1 SCARECROW-like 1 |
| OWA1   | 2014 | UIMiscanthus002771 1 108.3 99.3 112.4 Sobic.001G431500 AT5G16080 CXE17 carboxylesterase 17 |
| OWA2   | 2014 | UIMiscanthus088721 4 80.4 62.8 81.8 Sobic.002G351400 AT4G11650 OSM34 osmotin 34 |
| OWA3   | 2014 | UIMiscanthus048841 6 61.5 54.2 75.1 Sobic.003G237201 AT1G14920 RGA2 GRAS family transcription factor |
| OWA4   | 2014 | UIMiscanthus064552 8 66.1 55.9 76.4 Sobic.004G286600 AT1G204401.1 COR47 cold-regulated 47 |
| OWA5   | 2014 | UIMiscanthus086485 9 51.3 43.0 66.0 Sobic.005G103000 AT2G31230 ERF15 ethylene-responsive element binding factor |
| OWA6   | 2014 | UIMiscanthus036875 11 29.4 13.4 45.3 Sobic.006G098300 AT3G47700.1 CXE13 carboxylesterase 13 |

(Continues)
Within or near 25 of the 53 QTLs detected via joint-population analysis, we identified one or more candidate genes with previously described functions suggesting that they may be the causative gene(s) in these *Miscanthus* QTLs (Table 6). Additional candidate genes were also found via the single-population analyses (Supporting information Table S6). Candidate genes were identified within seven of the nine overwintering QTL. One QTL on *Miscanthus* LG 8 (QTL id: OWA4; Table 6) encompassed the cold tolerance gene, COR47, which was reported in *Arabidopsis thaliana* to protect thylakoid membranes during freezing by encoding dehydrin proteins, thereby resulting in improved cold tolerance (Bozovic, Svensson, Schmitt, & Kohn, 2013; Puhakainen et al., 2004). Upregulation of COR genes was also reported to increase freezing tolerance in perennial ryegrass (*Lolium perenne* L.) during cold acclimation (Zhang, Fei, Warnke, Li, & Hannapel, 2009). The overwintering ability QTL on LG 4 (QTL id: OWA2) contained the osmotin gene OSM34. Osmotin genes have been shown to induce proline accumulation that confers tolerance against both biotic and abiotic stresses in plants such as *Miscanthus*, tomato, strawberry, *Arabidopsis*, and olive trees (Abdin, Kiran, & Alam, 2010; D’Angeli & Altamura, 2007; Ings, Mur, Robson, & Bosch, 2013; Plażyek et al., 2014; Stavridou, Hastings, Webster, & Robson, 2017). A QTL on *Miscanthus* LG 11 (QTL id: OWA6) encompassed two plausible candidate genes, carboxylesterase 13 (CEX13) and WRKY transcription factor (WRKY2), both of which were reported to be responsive to cold stress in *Arabidopsis* (Seki et al., 2002), grape (*Vitis amurensis*) (Xin et al., 2013), and Peruvian lily (*Alstroemeria*) (Wagstaff et al., 2010). Carboxylesterase hydrolyzes a wide variety of carboxylic acid esters; although the functional details of carboxylesterase are still unknown (Xin et al., 2013), a member of this gene family in *Arabidopsis* was especially cold in Urbana, IL, with a minimum soil temperature at 10-cm of −6.2°C on 29 January 2014 and a minimum air temperature of −2.5°C on 6 January 2014. The overwintering ability QTL on LG 6 (QTL id: OWA3) contained one GRAS family transcription factor, RGA2, and this gene was reported to negatively regulate gibberellin biosynthesis and thereby indirectly control *Arabidopsis* growth and developmental process (Silverstone, Ciampaglio, & Sun, 1998); another GRAS family transcription factor, RGA1, was found in the spring...
regrowth QTL detected in the MapA population analysis on LG 14 (Supporting information Table S6). Such similar genes that underlay QTLs on different LGs could be paralogs of each other because of the recent genome duplication in *Miscanthus* (Dong, Liu, et al., 2018; Kim et al., 2014, 2012; Ma et al., 2012; Swaminathan et al., 2012). Ethylene-responsive element binding factor (ERF) proteins are transcription factors that signal plant response to environmental stresses (Licausi, Ohme-Takagi, & Perata, 2013; Müller & Munné-Bosch, 2015; Nakano, Suzuki, Fujimura, & Shinshi, 2006), and the overwintering ability QTL on LG 9 (QTL id: OWAS) contained ERF15. Within the overwintering QTL on LG 15 (QTL id: OWAS), we identified one WRKY transcription factor (WRKY46) and a second candidate gene lipid transfer protein 3 (LTP3). Overexpression of LTP3 has been shown to enhance freezing tolerance in *Arabidopsis* (Guo, Yang, Zhang, & Yang, 2013).

In addition to overwintering ability, we also detected QTLs for eight additional adaptation traits in the three interconnected F1 populations and found interesting candidate genes underlying these QTLs (Table 6). For spring regrowth, the QTL SRD1 contained the auxin-responsive GH3 gene, of which a homolog has been shown to regulate *Arabidopsis* growth and development processes, including shoot cell elongation, lateral root formation, and light response (Nakazawa et al., 2001). Autumn dormancy date QTL ADD2 contained the nonyellowing 1 gene (NYE1), which plays an important regulatory role in chlorophyll degradation during *Arabidopsis* senescence (Ren et al., 2007). The autumn dormancy date QTL ADD4 contained ABA-inducible BHLH-type transcription factor (AIB), which negatively regulates jasmonates (JA) signaling, thereby playing a pivotal role in fine-tuning of JA-mediated stress response and plant growth in *Arabidopsis* (Nakata et al., 2013). For May hardiness and May vigor, both of which had one QTL (QTL id: MH1, MVg1; Table 6) that co-localized on similar regions on LG 4, contained the SLOW GROWTH1 gene (SLO1), which was proposed to indirectly regulate *Arabidopsis* growth and development via affecting mitochondrial RNA editing and gene expression (Sung, Tseng, & Hsieh, 2010). The identification of candidate genes associated with significant QTL in three interconnected *Miscanthus* populations has generated testable hypotheses about gene regulation of key adaptation traits for this crop of emerging importance within the economically valuable Andropogoneae.

In summary, substantial variation was observed among and within three interconnected F1 *Miscanthus* populations for overwintering ability and eight other adaptation traits. Large differences among the three populations for overwintering ability indicated that it should be highly advantageous to select cold-tolerant genotypes as parents. Nevertheless, selection within the relatively nonhardy ornamental *M. sinensis* germplasm pool is expected to also produce substantial improvements in overwintering ability. Early spring regrowth date and early autumn dormancy date could be two important factors for indirectly selecting genotypes with strong overwintering ability. Furthermore, marker-assisted selection at the seedling stage should greatly improve the efficiency of selecting *Miscanthus* for overwintering ability and related adaptation traits, by reducing the need for multi-year field trials of this long-lived perennial. Moreover, the putative candidate genes identified in this study provide intriguing hypotheses and potential new lines of inquiry for determining the genetic and physiological mechanisms by which winter hardiness is conferred in *Miscanthus*.

**ACKNOWLEDGEMENTS**

This work was supported by the Energy Biosciences Institute, USDA HTACH project ILLU-802-311, and the DOE Office of Science, Office of Biological and Environmental Research (BER), grant no. DE-SC0006634. We thank Katarzyna Dubiel, Megan Swanson, Melina Salgado, and Tuo Shi for their assistance with DNA extraction. We thank the staff at the UIUC Energy Farm for maintaining the field trial and Pat Brown for providing *Psrl* adapters in RAD-seq library preparation.

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DONG et al.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Dong H, Liu S, Clark LV, et al. Winter hardiness of Miscanthus (II): Genetic mapping for overwintering ability and adaptation traits in three interconnected Miscanthus populations. GCB Bioenergy. 2019;11:706–726. https://doi.org/10.1111/gcbb.12587