Variability and adaptability of *Miscanthus* species evaluated for energy crop domestication

JUAN YAN*[1], WENLI CHEN†[1], FAN LUO†, HONGZHENG MA‡, AIPING MENG*, XINWEI LI*, MING ZHU*, SHANSHAN LI†, HAIFEI ZHOU†, WEIXING ZHU§, BIN HAN¶, SONG GE†, JIANQIANG LI* and TAO SANG†‖

*Key Laboratory of Plant Germplasm Enhancement and Speciality Agriculture, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan, Hubei 430074, China, †State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China, ‡College of Landscape Architecture, Beijing Forestry University, Beijing 100083, China, §Department of Biological Sciences, State University of New York, Binghamton, NY 13902, USA, ¶National Center for Gene Research & Institute of Plant Physiology and Ecology, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai 200233, China, ‖Department of Plant Biology, Michigan State University, East Lansing, MI 48824, USA

Abstract

A growing body of evidence indicates that second-generation energy crops can play an important role in the development of renewable energy and the mitigation of climate change. However, dedicated energy crops have yet to be domesticated in order to fully realize their productive potential under unfavorable soil and climatic conditions. To explore the possibility of domesticating *Miscanthus* crops in northern China where marginal and degraded land is abundant, we conducted common garden experiments at multiple locations to evaluate variation and adaptation of three *Miscanthus* species that are likely to serve as the wild progenitors of the energy crops. A total of 93 populations of *Miscanthus sinensis*, *Miscanthus sacchariflorus*, and *Miscanthus lutarioriparius* were collected across their natural distributional ranges in China and grown in three locations that represent temperate grassland with cold winter, the semiarid Loess Plateau, and relatively warm and wet central China. Evaluated with growth traits such as plant height, tiller number, tiller diameter, and flowering time, the *Miscanthus* species showed high levels of genetic variation within and between species. There were significant site × population interactions for almost all traits of *M. sacchariflorus* and *M. sinensis*, but not *M. lutarioriparius*. The northern populations of *M. sacchariflorus* had the highest establishment rates at the most northern site owing to their strong cold tolerance. An endemic species in central China, *M. lutarioriparius*, produced not only the highest biomass of the three species but also higher biomass at the Loess Plateau than the southern site near its native habitats. These results demonstrated that the wild species harbored a high level of genetic variation underlying traits important for crop establishment and production at sites that are colder and drier than their native habitats. The natural variation and adaptive plasticity found in the *Miscanthus* species indicated that they could provide valuable resources for the development of second-generation energy crops.

Keywords: biomass, cold tolerance, lignocellulosic energy crops, *M. lutarioriparius*, *M. sacchariflorus*, *M. sinensis*

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Correspondence: Jianqiang Li, tel. + 86 27 875 10330, e-mail: lijq@rose.whiob.ac.cn, Tao Sang, Department of Plant Biology, Michigan State University, East Lansing, MI 48824, USA, tel. + 1 571 355 4689, e-mail: sang@msu.edu

[1]Contributed equally to this work.

Introduction

Miscanthus is considered to be a promising candidate for second-generation energy crops (Clifton-Brown *et al.*, 2004, 2007; Karp & Shield, 2008; Oliver *et al.*, 2009). As a C4 perennial grass capable of producing high biomass in cool climates, *Miscanthus* is especially

suitable for growing in the temperate regions of the world (Beale & Long, 1995; Beale *et al.*, 1996; Naidu *et al.*, 2003; Wang *et al.*, 2008). Field trials of *Miscanthus × giganteus* conducted on Illinois cropland in the United States yielded more than 30 tons dry biomass ha^{-1} (Heaton *et al.*, 2008a). If this yield level is achieved for half of the 14.2 million hectares of the US cropland that is currently set aside for conservation, it can supply feedstocks for making 132 billion liters of ethanol (Somerville *et al.*, 2010). This could offset 20% of gasoline and 30% of CO_2 emission from petroleum used by the United States in 2008 (Heaton *et al.*, 2008a).

Miscanthus × giganteus is a sterile triploid hybrid between diploid *Miscanthus sinensis* and tetraploid *Miscanthus sacchariflorus* originated in Japan (Hodkinson *et al.*, 2002b). It was introduced to Europe as an ornamental in the 1930s and was tested as a potential energy crop half a century later (Long, 1987). The hybrid displayed a high photosynthetic rate and high water- and nutrient-use efficiencies, making it a candidate for second-generation energy crops with high net energy output (Beale & Long, 1997; Beale *et al.*, 1999; Clifton-Brown & Lewandowski, 2000a; Lewandowski *et al.*, 2000; Lewandowski & Schmidt, 2006; Heaton *et al.*, 2009). However, the productive potential of *M. × giganteus* is limited by its relatively weak abiotic stress tolerance. As demonstrated in the field trials in northern Europe, crop establishment from small rhizome cuttings suffered from a great loss over the first winter (Clifton-Brown & Lewandowski, 2000b). Subsequent studies showed that samples of the parental species, *M. sinensis*, from temperate Asia were more resistant to cold and drought stress than *M. × giganteus* (Clifton-Brown *et al.*, 2001, 2002; Lewandowski *et al.*, 2003), and had higher yield when grown under stressed conditions in Europe (Farrell *et al.*, 2006). Based on these observations, *Miscanthus* production models predicted that the improvement of abiotic stress tolerance would be key to realizing the yield potential, and improved crops capable of growing broadly in Europe could meet nearly 12% of the 27 EU states' primary energy needs by 2050 (Hastings *et al.*, 2009a, b).

These field trials conducted in the United States and Europe are consistent in suggesting that while *Miscanthus* has a potential to provide a major source of renewable energy, realization of this potential depends heavily on the ability to utilize land located in cold and dry climates. Furthermore, unlike the United States and EU, many countries in the world do not have extra cropland but have only land with poor soil quality and little irrigation resource for bioenergy production. Thus, sustainable bioenergy production in large scales requires domestication that develops new crops capable of producing sufficiently high biomass on marginal and degraded land with unfavorable climatic conditions (Jørgensen & Schwarz, 2000; Sang, 2011).

The first round of plant domestication that began approximately 10 000 years ago provided a stable source of food and laid the foundation of the civilization (Diamond, 2002). Food crop domestication was a process of largely unconscious selection for agricultural traits and could have lasted for centuries to millennia (Harlan, 1992; Purugganan & Fuller, 2009). To effectively mitigate climate change, however, this round of domestication of energy crops needs to be completed within a much shorter period of time, most likely years to decades. Under this time constraint, theoretical frameworks and experimental strategies must be developed to facilitate and accelerate the processes of the new crop domestication. The best way of doing this is to rely on what we have learned from studying the processes and mechanisms of the past crop domestications (Tang *et al.*, 2010).

A sufficient level of genetic variation in a crop species is critical for biotic and abiotic stress resistance and for continuous crop improvement (Tanksley & McCouch, 1997; Clifton-Brown *et al.*, 2008). Yet the domestication processes featuring strong artificial selection inevitably led to the reduction of genetic diversity from what existed in the wild progenitors (Buckler *et al.*, 2001; Zhu *et al.*, 2007; Burger *et al.*, 2008). Thus, taking a full advantage of variability and adaptability available in the wild progenitors would have been crucial to the success of crop domestication (Gepts, 2004; Doebley *et al.*, 2006).

Here we report the first study designed to exclusively evaluate variation and adaptation of *Miscanthus* species potentially serving as the wild progenitors of energy crops. The study was conducted in China, where the highest level of *Miscanthus* diversity including all high-biomass species is located (Chen & Renvoize, 2006; Clifton-Brown *et al.*, 2008). China possesses more than 100 million hectares of marginal and degraded land concentrated in northern and northwestern regions that could be made available for growing second-generation energy crops. This holds a potential of producing annually 1 billion tons of Miscanthus biofuel feedstocks, and makes northern and northwestern China a desirable place for experimenting energy crop domestication (Sang, 2011; Sang & Zhu, 2011).

This study evaluated three species, including *Miscanthus lutarioriparius*, the tallest growing *Miscanthus* species endemic to central China, and *M. sinensis* and *M. sacchariflorus*, the parental species of *M. × giganteus* (Hodkinson *et al.*, 2002a; Chen & Renvoize, 2006). A total of 93 populations of these species were collected across their distribution ranges in China and grown in three locations, Xilinhot of the Neimenggu Autono-

mous Region (XN), Qingyang of the Gansu Province (QG), and Jiangxia of the Hubei Province (JH). XN, located in the northern grassland of China, is the coldest and driest of all sites, while JH is the warmest and wettest site. QG is located on the Loess Plateau of northwestern China, one of the most eroded zone of the world. Multiple individuals of a population were sampled for the common garden experiment to evaluate within- and between-species variation. Morphological and physiological traits directly relevant to crop establishment and biomass yield were measured for the assessment of adaptive and productive potentials of the wild populations under distinct climatic conditions.

Material and methods

Plant material

Mature seeds of three *Miscanthus* species were collected across their distributional ranges in China in October and November 2008. A total of 93 populations were sampled for multiple-location trials, including 48 populations of *M. sacchariflorus*, 31 populations of *M. sinensis*, and 14 populations of *M. lutarioriparius* (Fig. 1; Table S1). For each population, seeds were collected from 20 to 30 well-spaced individuals, mixed in a paper bag, air-dried, and stored at room temperature.

In March 2009, about 200 seeds from each population were germinated in a Petri dish with wet filter paper at 25 °C. After 1 week, ~ 50 germinated seeds of a population were planted in float trays in the greenhouse of the Wuhan Botanical Garden. In May and June, young plants that were 15–20 cm tall were transplanted to three field sites (Fig. 1), including XN, QG, and JH. Transplanting started from south to north, in the sequence of JH, QG, and XN. Climatic data were obtained from the meteorological stations located nearest to the experimental sites. From May 2009 to October 2010, monthly average temperature, precipitation, and sunshine hours were shown in Fig. S1.

The rest of the germinated seeds were used for chromosome counting. Chromosome squashes were made using root tips of germinating seeds according to the protocols in Martin *et al.* (1994).

Multiple-location trials

The experiment used a completely random, two-way factorial design (species, site). A randomized table was generated for 93 populations each with 15 individuals. At each field site, seedlings were planted 1 m apart in a 60 × 24 m layout according to the order of individuals in the table. Thus, each field site was designed to grow ~1400 individuals with a planting density of
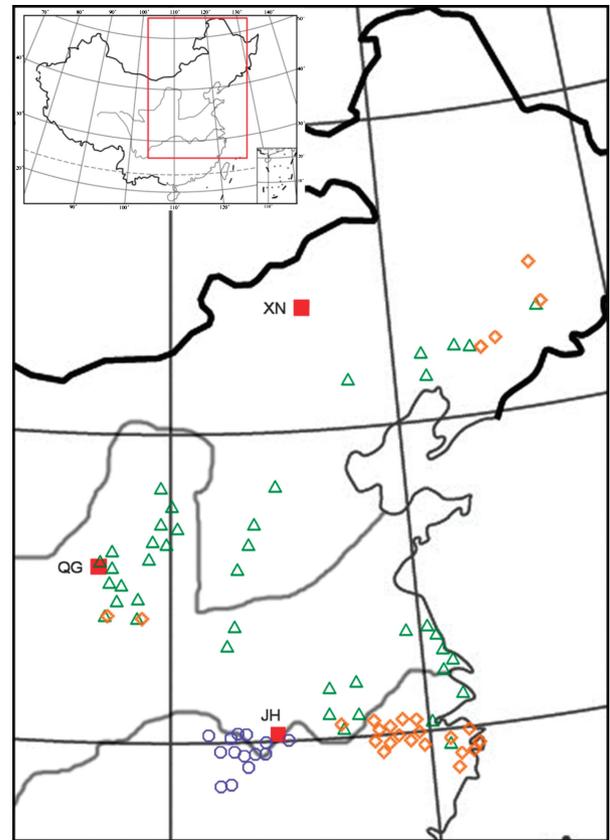
Fig. 1 Map of collection locations of populations studied in the common garden experiments. Triangle: *Miscanthus sacchariflorus*; diamond: *Miscanthus sinensis*; circle: *Miscanthus lutarioriparius*. Red squares indicate three field sites where the common garden experiments were conducted. XN, Xilinhot of the Neimenggu Autonomous Region; QG, Qingyang of the Gansu Province; JH, Jiangxia of the Hubei Province.

1 m² per individual. Seedlings were watered every other day during the first week of planting in 2009. Then watering was stopped at JH, but continued twice a week for 1 and 2 months at drier sites, QG and XN, respectively. In 2010, plants at QG and JH were not watered at all throughout the growing season. At XN where there was a severe summer drought, plants were watered twice in July. Fertilization was not applied during the experiment.

Plants that grew to the end of the 2010 season were all robust and thus considered to be established from the point of view of energy crop production. The establishment rate of a population, calculated as the ratio of the number of plants alive at the end of the 2010 growing season to the total number of seedlings planted in 2009, was used to evaluate the ability of the population to establish at the site. The proportion of a population that survived at the end of the 2009 growing season was defined as the seedling survival rate. This was deter-

mined by the ratio of survived plants counted in October 2009 to the total number of seedlings initially planted. The overwinter survival rate was used to evaluate the cold tolerance of a population, which was calculated as the ratio of the number of successfully sprouted plants in spring of 2010 to the number of living plants recorded in October 2009. The establishment rate was the product of the seedling survival rate and overwinter survival rate for the majority of the populations, except for those in which a small number of plants died following sprouting in 2010.

Several growth traits were measured for every individual grown at each site. Three morphological traits, plant height, tiller diameter, and tiller number, were measured at the end of the 2009 and 2010 growing seasons. Throughout the 2010 growing season, plant height was measured every 2 weeks at QG and JH, and every month at XN.

Plant height of an individual was measured from the ground to the top of the tallest tiller. The diameter of this tiller was measure at the position of ~10 cm aboveground. In 2010, when plant height was measured on the 2-week schedule, flowering time was recorded. An individual with at least one emerged inflorescence was considered to have flowered. At the end of the growing season in October or November of 2010, the tallest tiller of an individual was removed and brought to the laboratory where it was dried at 65 °C for 48 h and weighed for biomass.

Statistical analysis

ANOVAs were performed to test the effects of site and species on establishment rates, seedling survival rate, and overwinter survival rate. After a significant effect was detected for the interaction term (site × species), *post hoc* multiple comparisons of marginal means were carried out with the LSMEANS statement in the GLM procedure. Type-III sums of squares were used for the calculation of F-statistics. Principal component analysis was performed to determine the main influence on the establishment at XN and QG. PCA was conducted using the software SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

For growth traits (plant height, tiller diameter, tiller number, and flowering time) and biomass, because plants were considered to be established only after the winter of 2009–2010, our analyses were focused on the data collected at the end of the 2010 growing season. Further, because the majority of plants failed to survive over the winter at XN, comparisons were made primarily for plants grown at QG and JH. One population, Sa5, failed to establish at JH. So a total of 92 populations of the three species from both QG and JH were analyzed.

These traits were analyzed with nested ANOVAs that included species and sites as fixed effect and populations (nested within species) as a random effect. Means were analyzed with a mixed model and populations (nested within species) as a random effect. Type-III sums of squares were used to calculate F-statistics. *Post hoc* multiple comparisons of marginal means were performed with the LSMEANS statement in the GLM procedure. To estimate the variation among populations within species at both sites, ANOVAs were used to analyze each trait with populations and sites as fix effects.

At QG and JH, logistic curves were used to fit the growth as measured by plant height throughout the 2010 growing season. The growth rates were compared between the two sites by plotting the increase of plant height between two measurements against the time when the later measurement was taken. The flowering rate of a species was calculated as the ratio of flowered plants to the total number of growing individuals of the species at the 2-week recording time. Considering that a large proportion of plants did not flower at QG and *M. lutarioriparius* flowered during a very short period of time, the correlation analysis between flowering time and morphological traits was only performed for *M. sacchariflorus* and *M. sinensis* at JH. To test correlation between traits or traits and latitudes, Spearman's rank correlation coefficient was estimated using Proc CORR. ANOVA and Spearman's rank correlation were conducted using SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

Establishment

Plants that grew vigorously to the end of the 2010 growing season were considered to be established. The establishment rate of a population is approximately determined by the seedling survival rate in 2009 and the rate of surviving over the winter of 2009–2010. When compared among the three species at the three sites, the establishment rates were significantly different between species and were also significantly influenced by sites and site–species interactions (Table S2). When the species were compared at each site, *M. sinensis* and *M. lutarioriparius* had significantly lower establishment rates than *M. sacchariflorus* at XN, and *M. sinensis* established at a lower rate than the other two species at QG (Fig. 2a). At JH, the establishment rates were not significantly different among the three species.

At XN and QG, the overwinter survival rate appeared to be the primary contributor to the different establishment rates between species (Fig. 2a–c). This was
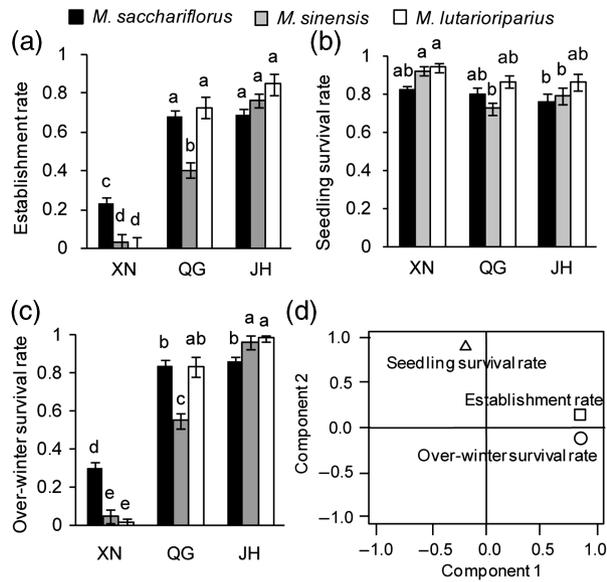
Fig. 2 Differences of (a) establishment rates, (b) seedling survival rates, and (c) overwinter survival rates between species and field sites. Different letters indicate significant differences at $P = 0.05$. Error bars indicate one standard error. (d) Principle component analysis indicates that overwinter survival rate contributed primarily to the establishment rate.

Fig. 3 Correlations between overwinter survival rates and the latitudes of population origin. (a), (c), and (e) *Miscanthus sacchariflorus* at XN, QG, and JH, respectively; (b), (d), and (f) *Miscanthus sinensis* at XN, QG, and JH, respectively. XN, Xilinhot of the Neimenggu Autonomous Region; QG, Qingyang of the Gansu Province; JH, Jiangxia of the Hubei Province.

supported by a principal component analysis indicating that establishment rates and overwinter survival rates were both more than 98% correlated with the first component (Fig. 2d). Thus, the differential establishment of these species at the two northern sites was due primarily to their different abilities of overwinter survival. At relatively warm JH, although the overwinter survival rate of *M. sacchariflorus* was lower than those of other two species, it apparently was not enough to cause a significantly lower establishment rate of the species.

In the coldest site, XN, overwinter survival rates were much lower than those at the other two sites (Fig. 2c; Fig. S1). Particularly, only small proportions of *M. sinensis* and *M. lutarioriparius* populations were able to survive the cold winter at XN. For *M. sinensis*, 25 of 31 populations failed to survive, and only two out of 14 *M. lutarioriparius* populations survived (Fig. S2). Of the eight populations of *M. sinensis* and *M. lutarioriparius* survived at XN, only two *M. sinensis* populations had overwinter survival rates higher than 40% and none of the populations had a survival rate higher than 60%.

In comparison, *M. sacchariflorus* had a much higher overwinter survival rate at XN, with 36 of 48 populations survived, of which 14 and 10 populations had overwinter survival rates higher than 40% and 60%, respectively (Fig. S2). In China, *M. sinensis* and *M. sacchariflorus* are distributed across wide latitudes. Cor-

relation analyses indicated that overwinter survival rates at XN had positive correlations with the latitudes of population origin for both species (Fig. 3a and b). Together, these results suggest that the northern populations of *M. sacchariflorus* have the strongest cold tolerance of all studied populations.

The relationship between overwinter survival rates and the latitudes of population origin was also examined at other two sites, QG and JH (Fig. 3c–f). A significantly positive correlation was found for *M. sinensis* at QG, suggesting that the southern populations of *M. sinensis* had trouble to survive the winter at this middle-latitude site. At JH, however, the correlation for *M. sinensis* was negative with a marginal significance. For *M. sacchariflorus*, negative correlations were found at both sites, indicating that southern populations of this species had higher overwinter survival rates at QG and JH. No significant correlation between any rates and the latitudes of population origin was

■ *M. sacchariflorus*   ▨ *M. sinensis*   □ *M. lutarioriparius*
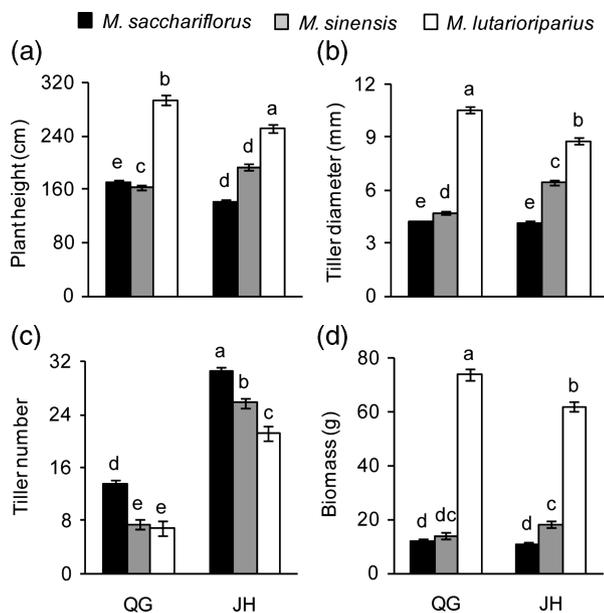
Fig. 4 Differences of (a) plant height, (b) tiller diameter, (c) tiller number, and (d) biomass between three species at QG and JH. Different letters indicate significant differences at $P = 0.05$. Error bars indicate 1 SE. QG, Qingyang of the Gansu Province; JH, Jiangxia of the Hubei Province.

found for *M. lutarioriparius*, which was most likely due to the narrow latitudinal distribution of the species.

Growth

For perennial herbaceous energy crops, the aboveground biomass of *Miscanthus* is harvested annually as biofuel feedstock. In this study, we measured four growth traits that were relevant to biomass yield. These included plant height, tiller diameter, tiller number, and flowering time. Because of the very low establishment rates of *M. sinensis* and *M. lutarioriparius* at XN, we focused our comparisons on data from QG and JH.

When the three species were compared at both sites, there was a significant site effect on between-species differences of plant height and tiller number but not tiller diameter (Table S3). For plant height and tiller diameter, there were significant site–species interactions. When we compared the three morphological traits between species at each site, we found that the majority of the comparisons differed significantly between species, except for plant height between *M. sinensis* and *M. sacchariflorus* at QG and tiller number between *M. sinensis* and *M. lutarioriparius* at QG (Fig. 4a–c).

Within-population sampling made it possible to evaluate between-population variation within a species. For all three species at both sites, there was a significant

within-species variation exhibited by almost all traits (Table 1). The only exception was the tiller number of *M. sinensis*. There was a significant site effect on all traits for each species. A significant site–population interaction effect indicated that three traits for *M. sacchariflorus* and plant height and tiller diameter for *M. sinensis* responded differently to sites depending on the population.

In 2010, the vast majority of plants flowered at JH, with flowering rates of 75.1%, 99.1%, and 93.4% for *M. sacchariflorus*, *M. sinensis*, and *M. lutarioriparius*, respectively. The flowering rates were much lower at QG, with 59.7%, 66.5%, and 7.2% for *M. sacchariflorus*, *M. sinensis*, and *M. lutarioriparius*, respectively (Fig. 5). At QG, the flowering rates of *M. sacchariflorus* are positively correlated to the latitudes of population origin ($r = 0.343$, $P = 0.017$), while the correlation is not significant for *M. sinensis* ($r = 0.147$, $P = 0.455$). All three species began to flower earlier at JH than QG; *M. sacchariflorus* and *M. sinensis* flowered as early as in June at JH but began to flower in August at QG (Fig. 5).

Considering that a large proportion of plants did not flower at QG, the further analyses of flowering time was conducted only for plants grown at JH. At JH, flowering time was significantly different between species ($P < 0.0001$). Within species, flowering was significantly different between populations of *M. sinensis* ($P < 0.0001$) and *M. sacchariflorus* ($P < 0.0001$) but not *M. lutarioriparius* ($P = 0.0635$).

Switching to reproductive growth slowed down vegetative growth and consequently affected the morphological traits. Indeed, we found significantly positive correlations between flowering time (days from January 1 of 2010) and plant height and tiller diameter for *M. sinensis* and *M. sacchariflorus* (Fig. 6a–d), meaning that later-flowered populations tended to have taller and thicker tillers. Flowering time was not correlated with tiller number (Fig. 6e and f). No significant correlation was found for any of these traits of *M. lutarioriparius* whose populations had a relatively narrow distribution (data not shown).

At JH, flowering time had a negative correlation with the latitudes of population origin for *M. sinensis* ($r = -0.564$, $P < 0.0001$), indicating northern populations of the species tended to flower earlier. For *M. sacchariflorus*, the negative correlation was not significant ($r = -0.239$, $P = 0.104$). This may be attributed to a high level of within-population variation in flowering time of the northern populations of *M. sacchariflorus* (Fig. S3).

In 2010, we monitored growth at QG and JH by measuring plant height every 2 weeks. The results showed that *M. lutarioriparius* grew much taller than other two species from the beginning to the end of the

Table 1 ANOVA of the effects of sites and populations on growth traits and their variation within species

Fixed effect	df	Plant height		Tiller diameter		Tiller number		Biomass	
		F	P-value	F	$\tilde{N}$-value	F	P-value	F	P-value
Miscanthus sacchariflorus									
Population	46	1.97	0.0002	9.11	<0.0001	2.53	<0.0001	5.59	<0.0001
Site	1	38.89	<0.0001	5.69	0.0173	203.36	<0.0001	6.59	0.0104
Site × Population	46	2.21	<0.0001	1.02	0.441	1.83	0.0008	1.77	0.0016
Error	778								
Miscanthus sinensis									
Population	30	7.8	<0.0001	5.69	<0.0001	0.42	0.9972	6.35	<0.0001
Site	1	35.25	<0.0001	65.35	<0.0001	178.12	<0.0001	9.12	0.0027
Site × Population	30	3.47	<0.0001	1.72	0.0116	1.11	0.3214	2.59	<0.0001
Error	446								
Miscanthus lutarioriparius									
Population	13	5.91	<0.0001	3.53	<0.0001	2.6	0.0021	2.93	0.0005
Site	1	36.38	<0.0001	28.59	<0.0001	230.56	<0.0001	3.98	0.0471
Site × Population	13	0.78	0.686	0.81	0.646	1.42	0.1496	0.86	0.5937
Error	257								
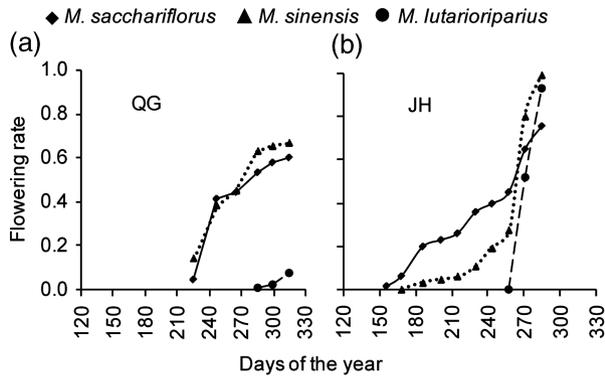
Fig. 5 Flowering phenology of three species at (a) QG and (b) JH. Proportions of populations of a species that flowered were recorded every 2 weeks in 2010. QG, Qingyang of the Gansu Province; JH, Jiangxia of the Hubei Province.

growing season at both locations (Fig. 7a). At JH, plants broke bud dormancy in early March of 2010 and all plants that survived the winter began to grow by the end of March. At the colder location, QG, the break of bud dormancy began early in April and was completed for all survived plants by the end of April. In terms of growth rates estimated by the 2-week increase in plant height, all three species had peaks of growth immediately following the break of dormancy in the spring (Fig. 7b and c). At QG, *M. lutarioriparius* had another growth peak in early August, which allowed its growth to surpass that at JH. The other two species had late growth peaks in September while the growth rate of *M. lutarioriparius* bottomed. At JH, only *M. sinensis* had a late growth peak in September.
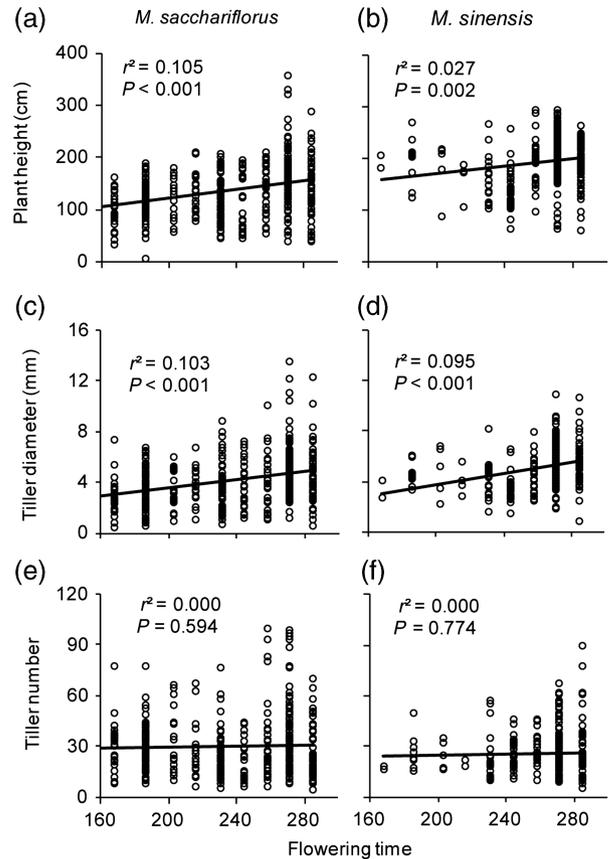
Fig. 6 Associations between flowering time and morphological traits of the two species at JH. (a), (c), and (e) plant height, tiller diameter, and tiller number for *Miscanthus sacchariflorus*, respectively; (b), (d), and (f) plant height, tiller diameter, and tiller number for *Miscanthus sinensis*, respectively. JH, Jiangxia of the Hubei Province.
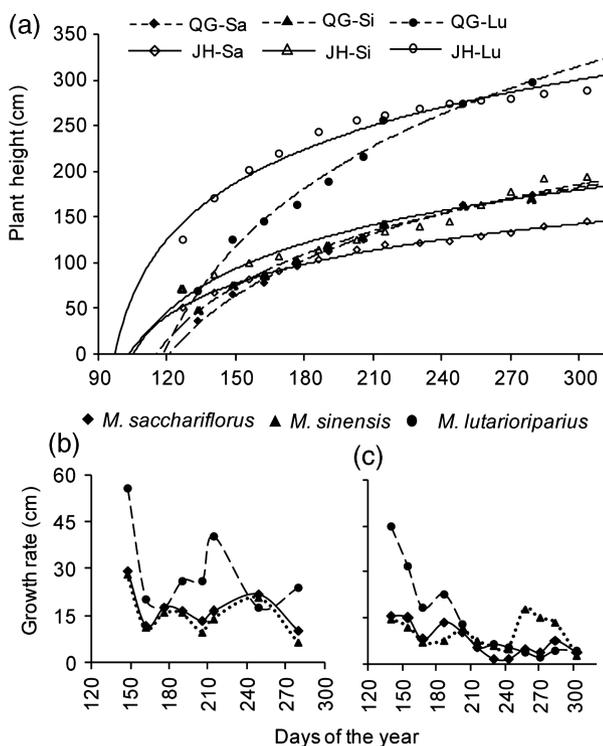
Fig. 7 Plant height monitored during the 2010 growing season. (a) Average plant height of a species calculated every 2 weeks following sprouting at QG and JH. Species abbreviations: Sa, *Miscanthus saccariflorus*; Si, *Miscanthus sinensis*; Lu, *Miscanthus lutarioriparius*. Logistic curves were used to fit the data. (b and c) Growth rates measured by the increase of plant height during 2-week intervals at QG and JH, respectively. QG, Qingyang of the Gansu Province; JH, Jiangxia of the Hubei Province.

Biomass

In addition to the growth traits, we measured tiller biomass (thereafter biomass) at QG and JH in order to better project the yield potential of the energy crops. When the three species were compared at both sites, biomass was significantly different between species and the differences were significantly influenced by sites (Table S4). When biomass was compared between species at each site, there was a significant difference for all comparisons except for between *M. saccariflorus* and *M. sinensis* at QG (Fig. 4d).

When biomass was compared within species at both sites, there was a significant between-population variation for each species (Table 1). The site effect on the within-species variation of biomass was significant for *M. saccariflorus* and *M. sinensis* but only marginally significant for *M. lutarioriparius*. The site–population interaction effect was responsible for biomass variation within *M. saccariflorus* and *M. sinensis* but not *M. lutarioriparius*.

We then analyzed relationships between biomass and the growth traits as well as the latitudes of population origin. At both sites, there were positive correlations between biomass and plant height and tiller diameter (Table S5), reflecting that taller and thicker tillers were heavier. In terms of correlation between biomass and tiller number, there were positive correlations for *M. saccariflorus* and *M. lutarioriparius* at JH. Positive correlation was found between biomass and flowering time for *M. saccariflorus* ($r = 0.240$, $P < 0.0001$) and *M. sinensis* ($r = 0.323$, $P < 0.0001$), but not for *M. lutarioriparius* ($r = 0.141$, $P = 0.088$).

Chromosome counting revealed that two populations of *M. saccariflorus*, Sa35 and Sa36, and one population of *M. lutarioriparius*, Lu02, were tetraploid ($2n = 76$), while the rest of the sampled populations of the three species were diploid ($2n = 38$). This extensive survey of chromosome numbers of *Miscanthus* species suggested that diploid populations were predominant in China, which contrasts to relatively high frequencies of tetraploid populations of *M. saccariflorus* found elsewhere such as in Japan (Hodkinson *et al.*, 2002b; Nishiwaki *et al.*, 2011).

Discussion

Estimating the genetic variability of wild progenitors, especially for adaptive and production-related traits, is a reasonable starting point of new crop domestication (Sang, 2011). By growing plants in the common garden, one can tease apart genetic from environmental effects on the phenotypic variation of adaptive traits. Regarding between-species differences in the three morphological traits, of 18 within-site pair-wise comparisons made at JH and QG, 16 were significantly different (Fig. 4a–c). This indicates that there is a high level of genetic variation underlying the morphological divergences between the species. This source of variation is valuable for crop domestication and can be utilized through selection on hybrids derived from crosses between wild species or between crops domesticated independently from different species, as was documented in the domestication of food crops such as rice (Sang & Ge, 2007a, b).

In addition to the differentiation between species, variation within species is another source of genetic diversity that can be even more directly incorporated into crops. By sampling within populations of each species, we were able to estimate variation between populations of a species (except the establishment rate for which we could not calculate within population variation). For the three morphological traits evaluated, a great deal of variation was detected within each species (Table 1).

Both *M. sacchariflorus* and *M. sinensis* are widely distributed, whereas *M. lutarioriparius* occupies a relatively narrow distributional range and ecological niche. It was recognized as a separate species from *M. sacchariflorus* based on its thicker and taller tillers and vigorous growth along seasonally flooded river banks and lake shores in central China (Chen & Renvoize, 2006). But in a recent taxonomic revision of the genus, *M. lutarioriparius* was reduced in rank and treated a subspecies of *M. sacchariflorus* (Sun *et al.*, 2010). However, our common garden experiments revealed that there were significant differentiations between these two species at all field sites, suggesting they are genetically diverged in these potentially adaptive traits. Thus, no matter which taxonomic status is more convenient in practice, our study shows that *M. lutarioriparius* consists of populations adapted to the distinct habitats and differentiated genetically from the populations of *M. sacchariflorus*.

Successful establishment under unfavorable field and climatic conditions is an important step that needs to be achieved at the early stage of energy crop domestication (Sang, 2011). The natural distributions of the *Miscanthus* species center on eastern and southern China, whereas the marginal and degraded land potentially available for growing energy crops is concentrated in northern and northwestern China (Sang & Zhu, 2011). One of the field sites, XN, is out of the natural distributional ranges of the three species. QG is at the western edge of the distribution of *M. sacchariflorus* and *M. sinensis* (Fig. 1).

Of the two components of the establishment rate, seedling survival rates during the first growing season were less variable between species and between sites (Fig. 2b). At the two northern sites with relatively cold winters, the ability of young plants to survive the first winter is the most serious limiting factor for crop establishment, as was shown in the previous studies in Europe (Clifton-Brown & Lewandowski, 2000b). In this study, the establishment rates were the lowest at XN for all three species, which is clearly attributed to the freezing kill during the cold winter at XN (Fig. S1). *M. sacchariflorus* was much more tolerant to the very low winter temperature at XN than other two species. Particularly, the northern populations of *M. sacchariflorus* were the most tolerant, which should be a result of their natural adaptation. This serves as a valuable genetic source for developing energy crops for growing in cold regions.

Southern populations of *M. sinensis* are the least cold-tolerant and had a significantly lower overwinter survival rate at QG than at JH (Fig. 2c). But the overwinter survival rates of other two species, *M. sacchariflorus* and *M. lutarioriparius*, were not significantly different between QG and JH. This suggests that in places of the Loess Plateau such as QG, *M. sacchariflorus*, *M. lutariori-*

parius, and the northern populations of *M. sinensis* had little trouble to survive through the winter.

At JH, the northern populations of *M. sacchariflorus* had lower overwinter survival rates than the southern populations of the species. This was probably influenced by factors other than cold stress. Northern populations of *M. sacchariflorus* did not grow as vigorously as southern populations at the stage of establishment, which was reflected in relatively low seedling survival rates of its northern populations at JH (Fig. S4).

Following the establishment, achieving higher biomass yield is the next important issue to consider for energy crop domestication. Yield is a complex trait influenced by many factors. Plant architecture and the duration and vigorousness of vegetative growth are of direct relevance to biomass yield. Our analyses indicated that tiller biomass was positively correlated with tiller length (plant height) and diameter that basically determined the tiller size. Biomass was positively correlated with flowering times of *M. sacchariflorus* and *M. sinensis*, and so were plant height and tiller diameter. Populations of these two species from higher latitudes flowered earlier at both QG and JH, which considerably slowed down their vegetative growth and subsequently limited the tiller size. Flowering too early is disadvantageous for energy crops because it cuts short the growing season permitted by local precipitation and temperature (Clifton-Brown *et al.*, 2008; Heaton *et al.*, 2008b).

In their natural habitats in China, *M. sacchariflorus* and *M. sinensis* flowered mostly in September and October (data not shown). When they were transplanted to QG and JH, a much wider range of flowering times was observed, from June to October in 2010 (Fig. 5). The earliest flowering plants were among the northern populations of *M. sacchariflorus* at JH, which could be due to a reaction to warmer temperature and/or different patterns of day length change. The degree of variation indicated that there was a high level of genetic variation controlling flowering time in *M. sacchariflorus* and *M. sinensis*, which may be a result of adaptation of the species to a wide range of latitudes (Clifton-Brown *et al.*, 2008; Stewart *et al.*, 2009). This variation can be a complicating factor for crop domestication on one hand, but an opportunity for optimizing the length of crop growing seasons on the other hand.

In addition to flowering time, the time of breaking bud dormancy and sprouting is another factor determining the length of the growing season of perennial plants. Although the *Miscanthus* species flowered earlier at JH than at QG, they sprouted earlier at warmer JH (Fig. 7a). At both sites, *M. lutarioriparius* flowered near the end of the growing season. While the relatively long growing season could have contributed to its high biomass, this was clearly not the only reason. At both

locations, *M. lutarioriparius* had higher growth rates than other two species. The natural populations of *M. lutarioriparius* are adapted to habitats with a higher water table and grow taller than other two species (Fig. S5), which implies that ample water supply might have been responsible to its higher productivity. However, the common garden experiments showed that *M. lutarioriparius* grew much bigger than other two species at either location, indicating that the higher productivity of the species is genetically based.

It is noteworthy that the plant height of *M. lutarioriparius* at QG was initially lower than that at JH, but surpassed it from July (Fig. 7a). At the end, the average biomass of the species at the drier site, QG, overtook that at JH, which was due primarily to a late growth peak at QG (Fig. 7b). It is unclear why *M. lutarioriparius* had the surge of growth at QG given that both temperature and precipitation were higher at JH and its native habitats alike (Fig. S1). One possibility is that a larger amount of solar radiation at QG than at JH might have played an important role in promoting the growth of the species (Dohleman *et al.*, 2009; Dohleman & Long, 2009). In any event, this adaptive plasticity makes *M. lutarioriparius* a valuable genetic resource for developing energy crops with high biomass yield for regions that are colder and drier than its natural habitats. Moreover, the sharp growth peak of *M. lutarioriparius* in early August was not found in other two species at QG, suggesting that they responded differently to environmental factors promoting growth. The genetic and physiological mechanisms underlying biomass production of the *Miscanthus* species awaits further investigation.

Acknowledgements

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