

Miscanthus sp.: Genetic Diversity and Phylogeny in China

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Abstract *Miscanthus* genetic resources are widely distributed throughout China. However, genetic studies on *Miscanthus* lagged far behind other crops (e.g., sorghum, maize). To establish the comprehensive genetics knowledge of *Miscanthus* in China, here we report the genetic and phylogenetic diversity of 174 domestic *Miscanthus* accessions, along with an external *Miscanthus* × *giganteus* control. Cytological observations and flow cytometry analyses indicated that there were two major *Miscanthus* cytotypes in China: diploid (86.86%) and tetraploid (12.57%) without triploid. A total of 108 polymorphic loci generated from 25 SSR primers were used to evaluate the genetic variation. Large variations in genetic similarity coefficients (GSCs), ranging from 0.08 to 0.97 with a mean value of 0.39, were observed between these *Miscanthus* accessions. Our phylogenetic data revealed that these accessions were clustered into four main clades: *M.* section *Miscanthus*, *M.* section *Diandranthus*, *M.* section *Triarrhena*, and hybrids. The average percentage of polymorphic loci (*P*), gene diversity (*H*), and Shannon's diversity index (*I*) among *Miscanthus* species are 70.93%, 0.22, and 0.34, respectively. These were consistent with the analysis of molecular variance (AMOVA) results, showing that 85% of

genetic variation was found within clades. This study investigated the clear phylogenetic relationship of *Miscanthus* species in China, which will be valuable for further utilization of the germplasm in genetic improvement and hybrid breeding of *Miscanthus*.

Keywords *Miscanthus* · Cytotype · Genetic diversity · Molecular phylogeny · SSR polymorphisms

Introduction

Miscanthus, a perennial C4 crop with rich lignocellulose, has received increasing attention worldwide for that it is potentially the next-generation renewable bioenergy resource. This biofuel crop is closely related to sorghum and sugarcane and is a member of the subtribe *Saccharinae*, tribe *Andropogoneae*, subfamily *Panicoideae*, and grass family of Poaceae (Hodkinson et al. 2002a). Of the 25 *Miscanthus* species worldwide, 14 of them are found in southern and eastern Asia as well as the Pacific Islands (Hodkinson et al. 2002b).

As one of the countries where *Miscanthus* originated, there is a wide range of genetically diversified germplasm in China (Sun et al. 2010; Zhang et al. 2013; Clark et al. 2014). To date, disagreements on how to classify *Miscanthus* species are still present. Early classifications were mainly based on morphological differences, resulting in two sections (*Miscanthus* and *Triarrhena*), along with a subsection (*Kariyasua*). *Miscanthus sinensis*, *Miscanthus sacchariflorus*, and *Miscanthus oligostachyus* were believed to belong to sections of *Miscanthus*, *Triarrhena*, and *Kariyasua*, respectively, and were further divided into 11 to 12 species (Hodkinson et al. 2002b, c; Sun et al. 2010). Chen and Renvoize (2006)

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identified seven species, *M. sinensis*, *M. sacchariflorus*, *Miscanthus lutarioriparius*, *Miscanthus floridulus*, *Miscanthus paniculatus*, *Miscanthus nepalensis*, and *Miscanthus nudipes* based on their paniculate inflorescences of racemes in mainland China. While *M. sinensis* and *M. sacchariflorus* are widely distributed in various locations in China, the remaining species are predominately identified in some specific ecological regions. For example, *M. paniculatus*, *M. nepalensis*, and *M. nudipes* are rarely found at high altitude areas (Chen and Renvoize 2006). Compared with *M. sacchariflorus*, *M. lutarioriparius* is endemic to central China where it can naturally grow vigorously up to 7 m in height with much thicker stems (Yan et al. 2012).

In general, *Miscanthus* can almost grow under any kind of environment (e.g., dry and unproductive mountainous regions, low wetlands, field footpaths, or seashores) due to its high water and nutrient use efficiency (Lewandowski and Schmidt 2006). Once established, it would show its high tolerance to various adverse conditions through the well-developed rhizome system. These favorable traits make *Miscanthus* an ideal lignocellulosic energy crop for most marginal land which are not ideal for grain or any crops of high economic value (Sang and Zhu 2010). Additional benefits include avoiding competition with food crops for fertile land and improving soil conditions and biological ecosystems.

Unlike many agronomic grain crops, most of *Miscanthus* germplasm remain uncharacterized and underutilized. Attention has greatly increased recently due to its huge potential as an important bioenergy crop (Chou 2009). The focus of *Miscanthus* studies over the last few years has been mainly limited to a very specific *Miscanthus* (e.g., *Miscanthus* × *giganteus*, *M. sinensis*). Cytologically, the base chromosome number of *Miscanthus* is $x = 19$, constituting its diploid $2n = 2x = 38$, triploid $2n = 3x = 57$, tetraploid $2n = 4x = 76$, and hexaploid $2n = 6x = 114$, respectively (Lafferty and Lelley 1994). Most *Miscanthus* species are self-incompatible and have a large complex genome with about 2.5 Gbp (Rayburn et al. 2009). Such genome size is equivalent to that of the maize genome but 6- and 20-fold of the *Oryza sativa* and *Arabidopsis thaliana* genomes, respectively.

Originated from Japan, *M. × giganteus* is recognized as a sterile triploid ($2n = 3x = 57$), and this vegetatively propagated *Miscanthus* is a hybrid of an allotetraploid *M. sacchariflorus* ($2n = 4x = 76$) × a diploid *M. sinensis* ($2n = 2x = 38$) (Linde-Laursen 1993; Lafferty and Lelley 1994; Hodkinson et al. 2002c). Compared with other types of *Miscanthus*, *M. × giganteus* has a number of advantages such as hybrid vigor, high yield, and wide range of adaptation to adverse environments (Cosentino et al. 2007; Christian et al. 2008). Currently, this is almost the only hybrid that has been widely grown in Europe and the USA for its biofuel purpose (Danalatos et al. 2007; Heaton et al.

2008). Clearly, the use of such narrow germplasm, i.e., only one hybrid, could result in a potential threat to the *Miscanthus* industry, due to its vulnerability to crop insects, diseases, and many other issues (Głowacka et al. 2015). Furthermore, the lack of genetic diversity studies on a wide range of *Miscanthus* germplasm may prevent the full use of other *Miscanthus* potentials.

Clark et al. (2014) recently studied the diversity and population structure on 703 *M. sinensis*, 12 *M. sacchariflorus*, 4 *M. floridulus*, 1 *M. oligostachyus*, as well as some hybrids by using SNP markers, and found south-eastern China to be the origin of *M. sinensis* populations. Studies on *Miscanthus* genetic diversity in South Korea (Qin et al. 2013; Yook et al. 2014), Japan (Shimono et al. 2013; Huang et al. 2014), and Europe (Slavov et al. 2013; Tang et al. 2015) have been mainly focused on a single species, *M. sinensis*, using limited samples from a restricted geographical distributions, while the similar results can also be found in China (Xu et al. 2013; Zhao et al. 2013; Nie et al. 2014).

Several studies on the ploidy level of *Miscanthus* have been reported. Lafferty and Lelley (1994) studied on four *Miscanthus* accessions and found diploid *M. sinensis*, triploid *M. × giganteus*, and both diploid and tetraploid *M. sacchariflorus*. Moon et al. (2013) studied ploidy diversity on 216 accessions collected from South Korea, including 49 *M. sinensis*, 164 *M. sacchariflorus*, and 3 *M. × giganteus*. In these accessions, diploid *M. sinensis* (21.29%), triploid *M. × giganteus* (1.39%), and both diploid (20.37%) and tetraploid (55.56%) *M. sacchariflorus* were identified. Li et al. (2013) studied 36 Chinese accessions including 12 *M. sinensis*, 14 *M. sacchariflorus*, and 10 *M. lutarioriparius*, and found that 2 *M. sacchariflorus* and 1 *M. lutarioriparius* were tetraploid (8.33%) and all of the others were diploid. A comprehensive genetic diversity study on a wide range of *Miscanthus* samples in China will be valuable for genetic improvement, breeding, and fundamental research in *Miscanthus* worldwide.

In this study, we collected 175 wild *Miscanthus* germplasm, including various species, varieties, and hybrids from different distinct geographic and ecological locations in China to reveal genetic diversity and understand the evolutionary relationships. We reported the results of cytological analyses on the germplasm to determine their ploidy levels and elucidated their phylogenetic relationships using the simple sequence repeat (SSR) markers. In contrast with previous work, our findings suggested that *M. lutarioriparius* was in fact a variant of *M. sacchariflorus* in China. The objectives of this study were to characterize the germplasm pool for wild *Miscanthus* germplasm in China, detect the ploidy levels of *Miscanthus*, and explore genetic diversity and phylogenetic evolution relationship for *Miscanthus*. This knowledge is essential for promoting genetic improvements and developing new germplasm and novel varieties of *Miscanthus*.

Materials and Methods

Miscanthus Germplasm Collections

According to the distribution of the genus *Miscanthus* which is recorded in Flora of China (Chen and Renvoize 2006), we collected 164 wild *Miscanthus* including 39 *M. sinensis*, 20 *M. floridulus*, 83 *M. sacchariflorus*, 16 *M. lutarioriparius*, 2 *M. nudipes*, and 4 hybrids that were widely distributed across the country. *Miscanthus* wild germplasm, naturally growing in various ecosystems of the most provinces with the exception of Xinjiang, Tibet, Qinghai, and Inner Mongolia in mainland China, were collected. Of all wild *Miscanthus* genetic resources collected, *M. sinensis* and *M. sacchariflorus* were widely distributed in China and exhibited strongest ecological adaptabilities. *M. sinensis* was found from latitude of 43.82° N of northeast China (e.g., Jilin Province) where temperature is very low to that of 19.45° N of southeast China where the temperature is very high (e.g., Hainan Province), and from longitude of 104° E to that of 127° E (altitude 27–1255 m). *M. sacchariflorus* was discovered from latitude of the northeast China 47.20° N (e.g., the north of Heilongjiang Province) to that of the southeast China 30.30° N (e.g., Hubei Province) and from longitude of 107° E to that of 128° E (altitude 3–1119 m). *M. floridulus* and *M. lutarioriparius*, on the other hand, were spread only within the region of southeast China. *M. lutarioriparius* is unique to China, which was found only in the middle and lower parts of Yangtze River.

M. nudipes grows only within the districts of Yunnan, Guizhou, and Chongqing where the altitude is about 1600–1700 m above the sea level. Interestingly, several hybrids derived from naturally crossing between *M. sinensis* and *M. lutarioriparius* and between *M. sinensis* and *M. sacchariflorus* had been identified and collected. Figure 1 and Online Resource 1 (ESM 1) illustrate all *Miscanthus* accessions collected, each of which with the detailed information on sample name, species, geographical location and altitude, etc. These locations represent many distinct ecological environments in China.

An external triploid *M. × giganteus* (kindly provided by the University of Illinois at Urbana-Champaign, USA) and ten domestic man-made interspecific hybrids (crossed between different *Miscanthus* species in China, Online Resource 1 (ESM 1)) were added into this study as controls. The collections of *Miscanthus* germplasm were planted in a 2.0 × 2.0 m plot, one replicate/genotype, on the Experimental Farm of Shandong Agricultural University, Tai'an, China, in March, 2011, and each genotype was also planted in the university greenhouse as a backup. No fertilizer was applied at any time during this study.

Ploidy Levels Determined by Cytological Observations and Flow Cytometry

Mitotic observations were performed based on the recent improved cytological techniques described by Kim et al. (2010). Specifically, root tips (3–4 cm long) from healthy

Fig. 1 Native distribution map of wild *Miscanthus* germplasm collected in China. Filled triangles indicate *Miscanthus sacchariflorus*, empty triangles indicate *Miscanthus sinensis*, filled squares indicate *Miscanthus lutarioriparius*, and empty squares indicate *Miscanthus floridulus*. *Miscanthus nudipes* and hybrids are marked by filled circles and empty circles, respectively

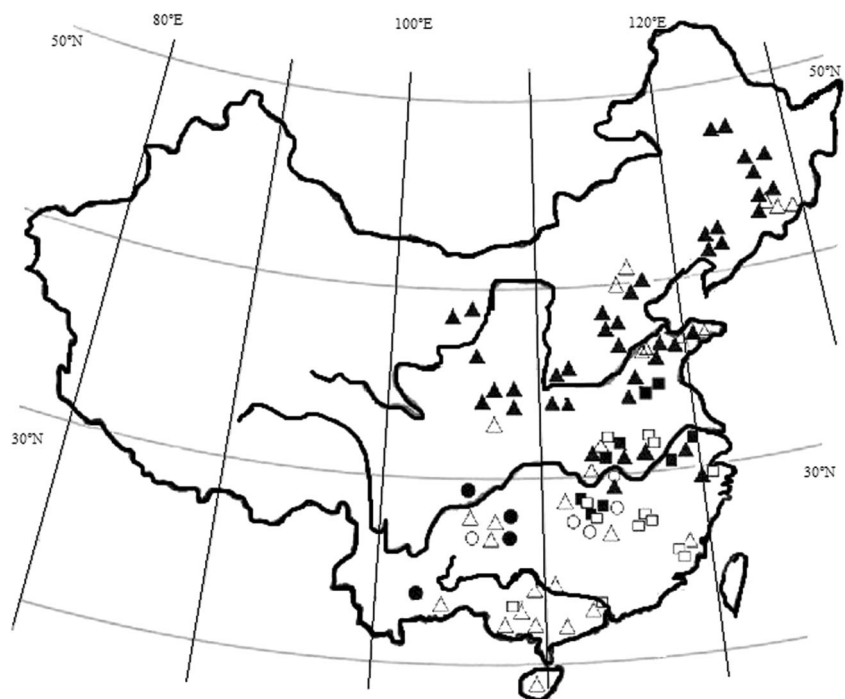


Table 1 Cytotypes of 174 *Miscanthus* accessions in China

Species	Diploid	Triploid	Tetraploid	Total
<i>Miscanthus sacchariflorus</i>	68		15	83
<i>Miscanthus lutarioriparius</i>	9		7	16
<i>Miscanthus sinensis</i>	39			39
<i>Miscanthus floridulus</i>	20			20
<i>Miscanthus nudipes</i>	2			2
Hybrids	14			14
<i>M. × giganteus</i> (control)		1		1
Total	152	1	22	175

Miscanthus accessions were taken at 8:30–9:30 am. Roots were then fixed with fresh fixative containing ethanol and acetic acid with 3:1 ratio (v/v) for 20 h and transferred to 70% ethanol to store at 4 °C. After the hydrolytic process, the roots were stained with carbol fuchsin. Active dividing cells with C-metaphase chromosomes were visualized and counted under an Olympus BX60 microscope (Olympus America Inc., Melville, NY, USA) and cells with clear chromosome images were recorded using a Nikon DIGITAL SIGHT DS-U3 camera (Nikon Instruments Inc., Tokyo, Japan).

Ploidy levels of various *Miscanthus* samples were determined based on the cytological data and nuclear DNA contents were measured by flow cytometry (PA model, Germany Partec) according to Rayburn et al. (2009). Briefly, 1–2 cm² fresh leaf tissue of each sample was submerged in 800 µl extraction buffer (13% hexylene glycol, 10 mM Tris-HCL, 10 mM MgCl₂) and 12 µl 25% Triton X-100 in a petri dish. The tissue was chopped into homogenized mixture and filtered. For DNA staining, 400 µl 4',6-diamidino-2-phenylindole (DAPI, 1 µg/ml) was added into the centrifuged nuclear pellet suspension. DNA contents of the diploid *Miscanthus* and triploid *M. × giganteus* were used as standards. Each sample was tested three times. Cytological results of all 175 *Miscanthus* samples examined in this study are listed in Table 1.

Genetic Diversity and Phylogeny Detected by SSR

To investigate genetic diversity and phylogenetic relationship, genomic DNA of 175 *Miscanthus* accessions were isolated using sodium dodecyl sulfate (SDS) method and diluted into 5 ng/µl as a working solution.

SSR markers were identified by searching the published genome database and EST sequences of *Miscanthus* (Ma et al. 2012; Barling et al. 2013) using MicroSATellite identification tool (MISA) and Primer5. Primers were synthesized by Dingguo Biotech Company (Beijing, China).

SSR markers were amplified in 10 µl PCR mixtures, each of which contained 10 ng of template DNA, 1× Green GoTaq buffer (Promega Corporation, Madison, USA) consisting of 2.0 mM MgCl₂, 0.15 µM of both reverse and forward primers (Beijing Dingguo Changsheng Biotechnology Co. LTD, Beijing, China), and 0.5 units of Taq polymerase (Thermo Scientific, Scoresby, Australia). The PCR reactions were performed on a Veriti 96-well thermal cycler (Applied Biosystems, USA). The reaction mixture of each sample was incubated at 94 °C for 5 min, followed by 35 cycles of 45 s denaturation at 94 °C, 30 s annealing at 55 °C, 45 s extension at 72 °C, and 7 min final extension at 72 °C. PCR products were separated on 8% polyacrylamide gels (PAG) (Amresco, Solon, OH, USA) and visualized by ethidium-bromide staining using Bio-Rad Gel Doc™ XR+ System (Bio-Rad Laboratories, Inc., California, USA).

Data Analysis

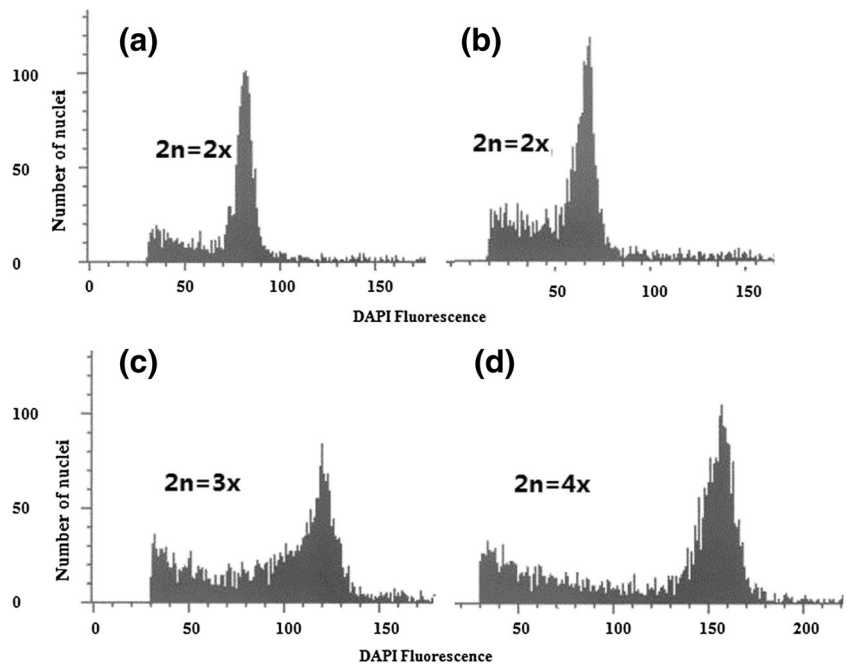
Out of the 40 SSRs, 25 primers generated scorable polymorphic amplicons in *Miscanthus*. Sequence information of the SSRs is listed in Online Resource 2 (ESM 1). These polymorphic SSR bands were converted to haplotype profiles as the required format of the program. Polymorphism information content (PIC) was calculated according to the method of Smith et al. (1997):

$$PIC_i = 1 - \sum P_{ij}^2$$

where P_{ij} is the frequency of the j th allele for the i th marker and is summed over n alleles. A binary matrix generated from coding 1 or 0 depending on presence or absence of each polymorphic SSR allele was used for calculating the genetic similarity coefficients (GSCs) by a Jaccard's similarity coefficient (Chae et al. 2014). The GSCs of these *Miscanthus* germplasm were employed for *Miscanthus* cluster classifications using neighbor joining (NJ). The NTSYS-pc version 2.02 (Rohlf 2000) was used to conduct both the phylogenetic tree construction with bootstrapping 1000 times and principal coordinate analysis (PCoA). The mean GS estimates of the inter- and intra-groups were calculated among all possible pairs of the collections belonging to each group.

To analyze phylogenetic relationships among various *Miscanthus* germplasm collected, 40 pairs of polymorphic SSR primers were initially used. Analysis of molecular variance (AMOVA) was performed using GenAlEx 6.5 (Peakall and Smouse 2012) to assess the genetic differentiation in the *Miscanthus* germplasm among and within species. Furthermore, the percentage of polymorphic loci (P), Nei's (1978) gene diversity (H), Shannon's information index of diversity (I), and F_{ST} (Wright 1965) among species was also calculated.

Fig. 2 Relative DNA contents revealed by flow cytometry analyses. **a** External diploid reference, CV = 4.62%; **b** diploid, CV = 4.66%; **c** triploid, CV = 3.20%; and **d** tetraploid, CV = 4.35%



Results

Cytotypes of Various *Miscanthus* Collections

Confirmation of Ploidy Levels by Flow Cytometry

Figure 2 illustrates the flow cytometric G0/G1 DAPI-stained nuclear DNA histograms generated by relative fluorescent intensities of representatives of diploids, triploids, and tetraploids, respectively. As shown in Fig. 2, the dominant peaks of a known diploid and a triploid *M. × giganteus* used as controls appeared at their respectively expected channels 80 and 120 (Fig. 2a, c). Clearly, samples showed their DNA peaks on the same channel as the diploid control were the results of diploids (Fig. 2b). Conversely, those with DNA peaks on channel 160 were tetraploids as their peaks had shifted to the right by another 80 channels (Fig. 2d). Similarly, DNA peaks positioned in between the diploids

and tetraploids on channel 120 were the results of triploids. Coefficients of variations (CVs) for the sample peaks in 175 *Miscanthus* germplasm were all within the normal range of 3 to 5% (Online Resource 1 (ESM 1)) (Doležel et al. 1998). The results of ploidy levels revealed by flow cytometry DNA data were consistent with those of cytological analyses.

Cytological Analyses

Chromosome counts revealed two major cytotypes, $2n = 2x = 38$ (Fig. 3a) and $2n = 4x = 76$ (Fig. 3c), along with *M. × giganteus*, $2n = 3x = 57$ (Fig. 3b). These data showed that, of the 175 *Miscanthus* samples examined, 152 (86.86%) were diploids ($2n = 2x = 38$), including 68 *M. sacchariflorus*, 9 *M. lutarioriparius*, 39 *M. sinensis*, 20 *M. floridulus*, 2 *M. nudipes*, and 14 hybrids. The second major cytotype with a total of 22 (12.57%) was tetraploids ($2n = 4x = 76$), 15 of which were *M. sacchariflorus*, and 7 were *M. lutarioriparius*.

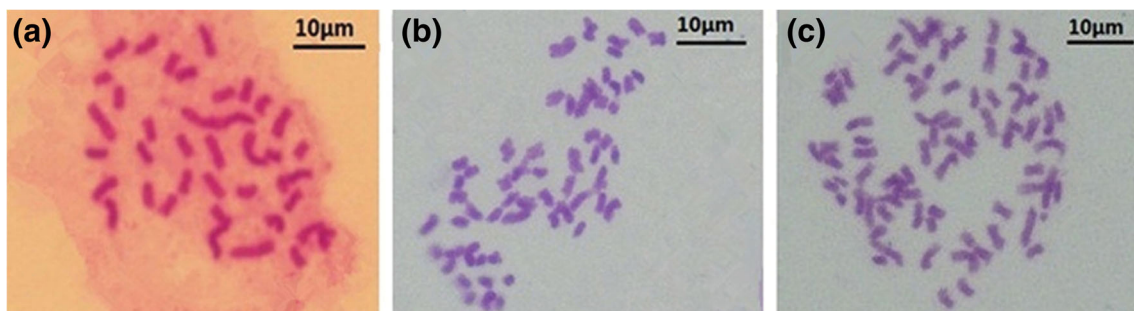


Fig. 3 Representatives of mitotic metaphase cells of various *Miscanthus* accessions. **a** Chromosome numbers of diploid ($2n = 2x = 38$), **b** triploid ($2n = 3x = 58$), and **c** tetraploid ($2n = 4x = 76$)

The triploid *M. × giganteus* control chromosome number was further confirmed to be $2n = 3x = 57$ (Table 1).

In the 83 *M. sacchariflorus* accessions, 15 (18.07%) were tetraploid, whereas 7 (43.75%) out of 16 *M. lutarioriparius* were tetraploids. No polyploid (neither triploid nor tetraploid) was identified among *M. sinensis*, *M. floridulus*, *M. nudipes*, and hybrids (Table 1). Tetraploids were found only from *M. sacchariflorus* and *M. lutarioriparius*. None of the accessions collected in this study was identified as triploid.

Genetic Diversity and Phylogeny Detection

Polymorphism of SSR Markers

Of 40 pairs of primers, only 25 resulted in amplified products showing clear abundant polymorphisms (Online Resource 2 (ESM 1)), and a total of 149 polymorphic bands identified from 175 accessions were amplified. The numbers of polymorphic bands varied with different SSRs, ranging from 3 to 9 and averaging 5.96 per locus. With the exclusion of those non-specific bands, a total of final 108 polymorphic bands (4.32/SSR) were used for the phylogenetic evaluation of the collected *Miscanthus* samples (Online Resource 2 (ESM 1)).

In general, PIC reflect the allele diversities and frequencies within the examined materials. In this study, ESSR13 showed the lowest PIC value of 0.41, whereas the highest PIC value for GSSR66 was 0.81, with an average of 0.63 among all loci (Online Resource 2 (ESM 1)).

Genetic Variations and AMOVA Analysis

The GSCs were calculated based on SSR polymorphisms present in these *Miscanthus* germplasm, ranging from 0.08 to 0.97, with an average GSC 0.39 (Online Resource 3 (ESM 1)). Two diploid genotypes M157 (*M. sacchariflorus* collected from Beijing, China) and M324 (*M. sinensis* collected from Sichuan, China) showed the lowest GSC 0.08, whereas two tetraploid cytotypes M049 and M050 (*M. sacchariflorus* collected from Henan, China) gave rise to the highest GSC 0.97 (Online Resource 3 (ESM 1)).

These GSC data were the clear indications of the presence of abundant genetic variations in these *Miscanthus* in China.

As indicated in Online Resource 4 (ESM 1), both *M. sinensis* (GSCs from 0.26 to 0.90) and *M. sacchariflorus/M. lutarioriparius* (GSCs from 0.34 to 0.97) showed higher intra-species genetic diversity than that of other species. Similarly, hybrids also exhibited high genetic variations (GSC 0.34 to 0.93). However, little variation was detected within *M. nudipes*, perhaps largely due to the very limited numbers (only two) collected from only two locations. Medium intra-species genetic diversity with GSCs from 0.31 to 0.86 was detected in *M. floridulus*. The mean GSC (0.51) among *M. floridulus* was higher than that among *M. sinensis* (0.49) (Online Resource 4 (ESM 1)).

Data listed in Table 2 indicated that the highest interspecies GSC between *M. sinensis* and *M. floridulus* is 0.37 and the lowest interspecies GSC between *M. floridulus* and *M. sacchariflorus/M. lutarioriparius* is 0.22. These results not only revealed clear genetic variations but also showed evolutionary relationships among these germplasm resources. Interestingly, comparing the GSCs of the hybrids to those detected between *Miscanthus* species, the highest GSC (0.43) appeared in-between the hybrids and the two species *M. sacchariflorus/M. lutarioriparius*, demonstrating their close evolutionary relationship. The hybrids have close genetic distance to the *M. sacchariflorus/M. lutarioriparius* and this may be due to that *M. sacchariflorus/M. lutarioriparius* is the dominant population in China.

The AMOVA analysis (Table 3) showed that genetic variations were significant in both within and between *Miscanthus* species, and the genetic variation of within species (85%) was substantially greater than that of between species (15%). The global genetic differentiation between these species was relatively weak but statistically significant ($F_{ST} = 0.16$, $P < 0.001$). The highest genetic differentiation was between *M. floridulus* and *M. sacchariflorus/M. lutarioriparius* ($F_{ST} = 0.40$, $P < 0.001$), while the lowest was between *M. sacchariflorus/M. lutarioriparius* and hybrid ($F_{ST} = 0.17$, $P < 0.001$). The indirect estimator of gene flow (N_m) among species was 2.75. According to the three main genetic diversity parameters including Nei's genetic distance

Table 2 Average GSC between *Miscanthus* species

GSC	<i>M. section Miscanthus</i>		<i>M. section Diandranthus</i>	<i>M. section Triarrhena</i>
	<i>Miscanthus floridulus</i>	<i>Miscanthus sinensis</i>	<i>Miscanthus nudipes</i>	<i>Miscanthus sacchariflorus/Miscanthus lutarioriparius</i>
<i>M. sinensis</i>	0.37			
<i>M. nudipes</i>	0.30	0.36		
<i>M. sacchariflorus</i> and <i>M. lutarioriparius</i>	0.22	0.23	0.26	
Hybrids	0.31	0.34	0.33	0.43

Table 3 Summary statistics of AMOVA analysis in *Miscanthus* germplasm

Source	df	SS	MS	Est. Var.	% Var.
Among species	4	60.37	15.09	0.47	15%**
Within species	170	438.67	2.58	2.58	85%**
Fixation Index	FST, 0.16 ($P < 0.001$)				

df degrees of freedom, SS sum of squared observations, MS mean of squared observations, Est. var. estimated variance, % Var. percentage of total variance

** $P < 0.01$, significance test of difference

(H), Shannon's information index of diversity (I), and percentage of polymorphic bands (P), the *Miscanthus* germplasm genetic resources in China have a varied genetic diversity. Among the *Miscanthus* species, the P value ranged from 16.67% (*M. nudipes*) to 94.44% (*M. sacchariflorus*/*M. lutarioriparius*), with an average of 70.93%. The H value ranged from 0.08 to 0.30, with an average of 0.22 at the population level. While the variation trend of I value was similar to the other two parameters, with an average of 0.34 (Table 4).

Cluster Analysis

A phylogenetic tree was constructed based on the GSC data (Fig. 4). In this tree, 175 samples were divided into four major clades (I, II, III, and IV) (Fig. 4). The major clades (I, II, and III) represented *M.* section *Miscanthus*, *M.* section *Diandranthus*, and *M.* section *Triarrhena*, respectively, while clade IV included all hybrids. Clade I consists of two sub-branches, corresponding to the two species in *M.* section *Miscanthus*: *M. sinensis* and *M. floridulus*, clade II includes *M. nudipes*, clade III represents the two species (*M. sacchariflorus* and *M. lutarioriparius*) combined in *M.* section *Triarrhena*, and clade IV covers all hybrids, including the external triploid *M. × giganteus* control (Fig. 4 and Online Resource 1 (ESM 1)).

To characterize the genetic variation of these *Miscanthus* germplasm, a three-dimensional PCoA (Fig. 5) were

constructed. All of 175 materials were scattered in a specific determined space and clearly divided into four groups (I, II, III, and IV), which were corresponding to the groups in the phylogenetic tree (Figs. 4 and 5). Clusters I, II, III, and IV were occupied by *M. sinensis* and *M. floridulus*, *M. nudipes*, *M. sacchariflorus* and *M. lutarioriparius*, and hybrids, respectively. The first, second, and third principal coordinates, termed PC1, PC2, and PC3 explained 22.68, 4.43, and 3.54% of the total variations, respectively. Three species, *M. sinensis*, *M. nudipes*, and *M. floridulus* were clustered together along dimension of PC1. *M. floridulus* was genetically separated from *M. sinensis* and *M. nudipes* along dimension of PC2 and PC3. *M. lutarioriparius* were located at the lower position of the PC3 axis within the *M.* section *Triarrhena* group (Fig. 5).

The hybrids in cluster IV were distributed between *M.* section *Triarrhena* and *M.* section *Miscanthus* (Fig. 5). Triploid hybrid *M. × giganteus* was also included in cluster IV. *M. × giganteus* was believed to be a hybrid of a tetraploid *M. sacchariflorus* ($2n = 4x = 76$) × a diploid *M. sinensis* ($2n = 2x = 38$) (Linde-Laursen 1993).

Discussion

Miscanthus Genetic Diversity in China and Their Evolutionary Relationships

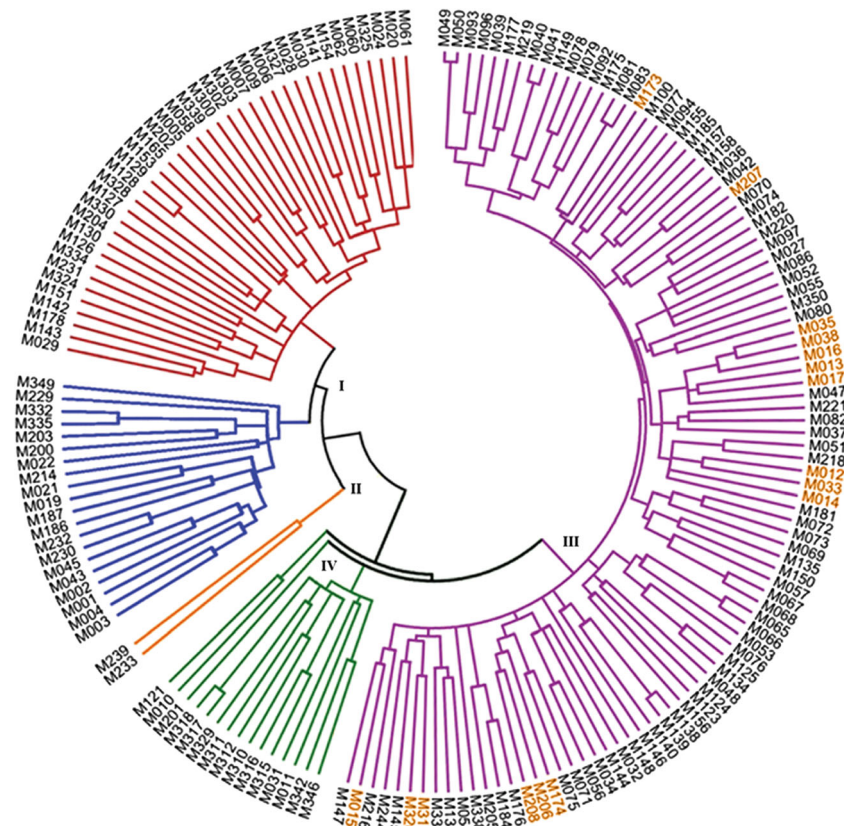
The data presented in this report are the result of comprehensive analyses using the techniques of cytology, flow cytometry, SSR molecular markers, and phylogenetics. Our cytological studies indicated that most of *Miscanthus* germplasm in China, including all natural hybrids, were diploid, along with some specific regional distributed tetraploids of *M. sacchariflorus* and *M. lutarioriparius*. Our results are in good agreement with those reported by Li et al. (2013), who studied 36 populations of three *Miscanthus* species from China: *M. lutarioriparius*, *M. sacchariflorus*, and *M. sinensis* by flow cytometry technique. Using the same method, similar conclusions from investigating the 216

Table 4 Genetic diversity of *Miscanthus* germplasm in China

Species	Sample Size	H	I	P value (%)
<i>Miscanthus floridulus</i>	20	0.24	0.36	73.15
<i>Miscanthus sinensis</i>	39	0.30	0.46	92.59
<i>Miscanthus sacchariflorus</i> / <i>Miscanthus lutarioriparius</i>	99	0.24	0.38	94.44
Hybrids	15	0.24	0.37	77.78
<i>Miscanthus nudipes</i>	2	0.08	0.12	16.67
Mean		0.22	0.34	70.93
SE		0.01	0.01	14.17

H Nei's gene diversity, P percent age of polymorphic bands, I Shannon's information index of diversity

Fig. 4 Dendrogram constructed with a neighbor-joining clustering algorithm from the pairwise matrix of genetic similarities among 175 *Miscanthus* germplasm accessions with bootstrapping 1000 times. *Miscanthus floridulus* (blue), *Miscanthus sinensis* (red), *Miscanthus nudipes* (yellow), hybrids (green), *Miscanthus sacchariflorus* (purple, black letters) and *Miscanthus lutarioriparius* (purple, orange letters)



South Korea *Miscanthus* germplasm with diploid (20.37%) and tetraploid (55.56%) *M. sacchariflorus* have recently been reported (Moon et al. 2013).

Our SSR and phylogenetic analyses revealed that there were relatively high genetic variations within each species and among various species in the Chinese genus *Miscanthus*. These data demonstrated that high genetic similarity is present in the *Miscanthus* germplasm in China. Early studies on 260 individuals of *M. sinensis* collected from 24 natural populations in Southwest China showed that inter- and intra-species genetic similarities ranged from 0.57 to 0.97 with an average of 0.66 (Nie et al. 2014). Similarly, genetic similarities of 69 *M. sinensis* and *M. sacchariflorus*/*M. lutarioriparius* derived from South Korea, Japan, and China were from 0.34 to 0.86 (Yook et al. 2014).

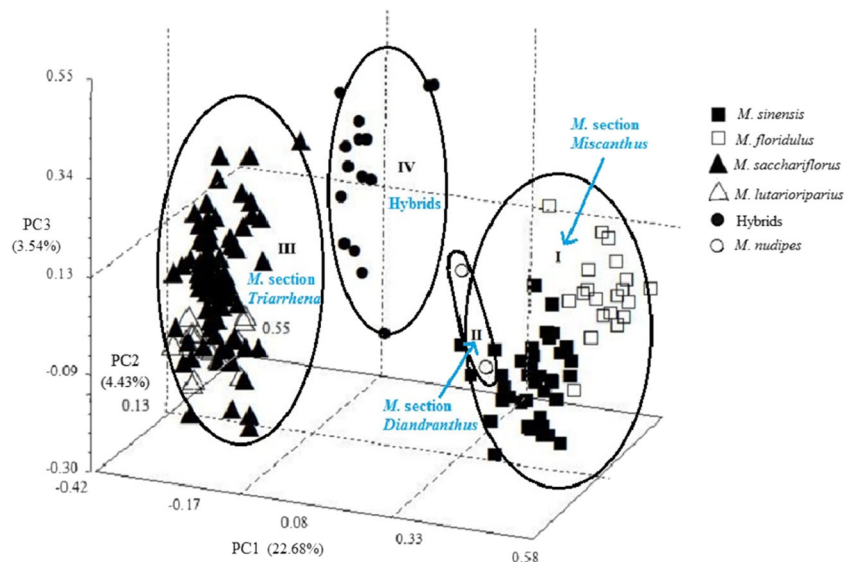
Our interspecific phylogenetic data showed that the least genetic similarity was between *M. floridulus* and *M. sacchariflorus* (GSC 0.22, Table 2), and the closest interspecific relationship was between *M. sinensis* and *M. floridulus* (GSC 0.37, Table 2). In the study of Clark et al. (2014), *M. floridulus* was also genetically close to *M. sinensis*, whereas *M. sacchariflorus* was clearly distinct from *M. sinensis*. Furthermore, the highest GSC (0.43, Table 2) appeared in between the hybrids and the two species *M. sacchariflorus* and *M. lutarioriparius*, demonstrating that hybrids were more evolutionarily related to *M. sacchariflorus*/

M. lutarioriparius than to other species, which might be due to its self-incompatibility, high heterozygosity, and complex genome in the genus *Miscanthus*.

Classifications of *Miscanthus* Germplasm in China

By combining cytological and molecular biological techniques, we successfully evaluated the genetic evolutionary relationship of the 174 wild *Miscanthus* germplasm collected from various ecological districts in China. Our results suggested that the use of molecular phylogenetic analysis enabled us to distinguish different species and identify the naturally derived hybrids. The examined *Miscanthus* germplasm in this study could be divided into three sections: *M. section Miscanthus*, *M. section Diandranthus*, and *M. section Triarrhena*. These three sections include five main *Miscanthus* species, of which *M. sinensis* and *M. floridulus* belong to *M. section Miscanthus*, *M. nudipes* is part of *M. section Diandranthus*, and *M. sacchariflorus* and *M. lutarioriparius* are members of *M. section Triarrhena*. Additionally, our ten man-made interspecific hybrids, four naturally derived hybrids, and the external control *M. × giganteus* were gathered closely in the phylogenetic tree (Fig. 4). On the other hand, Chae et al. (2014) suggested that *Miscanthus* accessions fell into five taxonomic groups, including the existing taxonomic section *Miscanthus*, diploid and tetraploid *Miscanthus sacchariflorus*, and a fourth (*M. × giganteus*) and

Fig. 5 Genetic associations among the 175 *Miscanthus* accessions revealed by principal coordinate analysis (PCoA). Three-dimensional PCoA for four *Miscanthus* sections



fifth group (*Miscanthus* “purpurascens”). Furthermore, AMOVA analysis agreed with the clustering pattern, the NJ tree, and the genetic distance measurements among the four clades including five main *Miscanthus* species and the hybrids, suggesting the accuracy and reliability of our phylogenetic analyses.

It is noteworthy that the two species *M. sacchariflorus* and *M. lutarioriparius* in *M. section Triarrhena*, could not be categorized into two independent sub branches. With regard to the considering morphological similarities (e.g., similar stamens and awnless spikelets per flower) between two species except the major difference in plant height (Chen and Renvoize 2006; Yan et al. 2012), it seems that *M. lutarioriparius* is just a variant of *M. sacchariflorus* species. This suggestion is not in agreement with the result of Chae et al. (2014) who reported that diploid and tetraploid *M. sacchariflorus* were taxonomically different, the latter more closely related to *M. lutarioriparius*. However, our speculation is similarity to that of Sun et al. (2010) who concluded that *M. lutarioriparius* was most likely to be a variant of the same species, *M. sacchariflorus*, since plant heights were the only difference between the two.

Miscanthus Tetraploids and Their Regional Distributions

Through analyzing distribution and localization of ploidy level of each accession, we found that tetraploids of *M. sacchariflorus* and *M. lutarioriparius* were geographically distributed in the Middle East of China (Latitude 28° 16' N–37° 30' N, Longitude 122° 18' E–122° 18' E, Online Resource 1 (ESM 1)). While both diploid and tetraploid coexisted in these regions, diploids were also identified in other parts of China.

Plant whole genome duplication (WGD) has been recognized to be one of the major driving forces for plant evolution

and speciation, and almost all angiosperms had undergone one or several times of WGDs during their long evolutionary history (Mallet 2007; Renny-Byfield et al. 2010). Maize, one of the most important economic crops, is a paleopolyploid, a result of at least three times of WGDs (Gaut and Doebley 1997; Blanc and Wolfe 2004; Swigonova 2004). *Miscanthus* belongs to subtribe sugarcane, and the ancestor of the sugarcane subtribe was separated from the sorghum subtribe about 3.8–4.6 million years ago (Kim et al. 2014b). A recent study on genome comparison has demonstrated the high similarities between *M. sinensis* and *Sorghum bicolor*, suggesting that *Miscanthus* was a result of WGD of the ancestral *Saccharinae* chromosome number $x = 10$, with a single chromosome fusion, leading to the odd base chromosome number $x = 19$ (Swaminathan et al. 2012; Kim et al. 2014a). Tetraploidization of *Miscanthus* occurred most likely after its divergence from its closely associated sugarcane clade (Swaminathan et al. 2012). However, the mechanism underlying the occurrence of the tetraploids of *M. sacchariflorus* and *M. lutarioriparius* in China remains to be further elucidated.

In conclusion, this study described, for the first time, cytological analyses, the genetic diversity, and phylogenetic evolution of various *Miscanthus* species and hybrids located in China. Abundant genetic variations were detected within and among *Miscanthus* species. Another significant achievement of this study is the discovery of *M. sacchariflorus* and *M. lutarioriparius* tetraploids that are localized in several provinces where climates are similar. Furthermore, our phylogenetic data suggest that *M. lutarioriparius* is in fact a variant of *M. sacchariflorus*. These results will be undoubtedly valuable for further genetic breeding, fundamental research, and ultimate utilization of the rich *Miscanthus* genetic resources not only in China but also elsewhere in the world.

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