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RESEARCH

The Discovery of Natural *Miscanthus* Accessions Related to *Miscanthus* × *giganteus* Using Chloroplast DNA

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ABSTRACT

It is essential to enlarge the pool of varieties of the biomass crop *Miscanthus* to support the increase in its cultivation area, and several natural species from eastern or southeastern Asia could be of interest. Our main study objectives were: (i) to investigate the frequency spectrum of the haplotypes of natural Chinese accessions and their geographic distribution in China, and (ii) to identify the Chinese chloroplast genomes related to the maternal genomes of cultivated European varieties. We studied 21 clones cultivated in Europe and 44 wild Chinese accessions from 10 Chinese provinces covering four species of *Miscanthus*: *M. sacchariflorus* (Maxim.) Hack., *M. sinensis* Andersson, *M. floridulus* (Labill.) Warb. ex K. Schum. & Lauterb., and *M.* × *giganteus* J.M. Greef & Deuter ex Hodkinson & Renvoize. We used chloroplast DNA from sugarcane (*Saccharum officinarum* L.) due to its taxonomic relationship with *Miscanthus* and designed primers using the large single-copy region of the chloroplast genome. The polymorphisms belonged to noncoding and coding regions and were substitutions that corresponded to single nucleotide polymorphisms. Haplotypes were then determined, enabling the investigation of the haplotype frequency spectrum and the geographic distribution of the accessions in China. Furthermore, the maternal genome of *M.* × *giganteus* appears related to some Chinese *M. sacchariflorus* clones. The corresponding geographic native area of these wild clones could be of interest to enlarge the genetic variability for the breeding of new interspecific hybrids.

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Abbreviations: ADAS, Agriculture Development and Advisory Service; cpDNA, chloroplast DNA; cpSSR, chloroplast microsatellite marker; INRA, Institut National de la Recherche Agronomique; PCR, polymerase chain reaction.

The genus *Miscanthus* belongs to the family Poaceae, tribe Andropogoneae, subtribe Saccharineae, and its members are closely related to sugarcane (*Saccharum officinarum* L.) (Hodkinson et al., 2002b). *Miscanthus* sensu lato (s.l.) comprises >20 species (Hodkinson et al., 2002a), whereas *Miscanthus* sensu stricto (s.s.) contains approximately 12 species (Clifton-Brown et al., 2008). There is no consensus to date with regard to the definition of *Miscanthus* (s.l. or s.s.), the taxonomic system to be used, or the number of species, subspecies, and forms recognized. This situation may be due to the existence of natural interspecific hybrids and variable levels of ploidy. According to Deuter (2000), the ploidy level of *Miscanthus sacchariflorus* (Maxim.) Hack. varies from diploid to hexaploid, diploid in China and tetraploid in Japan. For the tetraploid form (4*x*), *M. sacchariflorus* is considered allotetraploid, presumably AABB from *M. sinensis* Andersson and *M. sacchariflorus* parents as described in Hodkinson et al. (2002c)

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and verified previously via bivalent:disomic chromosome pairing (Adati and Shiotani, 1962). Moreover, Ma et al. (2012) demonstrated that the diploid *M. sinensis* is tetraploid origin consisting of two subgenomes. Several species occur naturally in eastern and southeastern Asia (Clifton-Brown et al., 2008), Africa, and the Himalayas (Hodkinson et al., 2002a).

Miscanthus has been identified as a good candidate biomass crop (Heaton et al., 2008). An ornamental plant from the genus *Miscanthus* was introduced to Denmark from Japan in 1935 by the Danish nurseryman Aksel Olsen (Greef and Deuter, 1993). The plant was later named *M. sinensis* 'Giganteus'. Linde-Laursen (1993) found that this triploid *M.* × *giganteus* clone in Europe was the same species as *M.* × *ogiformis* Honda described in Japan. This natural triploid species is believed to be an interspecific hybrid between an allotetraploid *M. sacchariflorus* and a diploid *M. sinensis* (Hodkinson et al., 2002c). Moreover, Hodkinson et al. (2002c) showed that the maternal lineage of *M.* × *giganteus* was *M. sacchariflorus* using chloroplast DNA (cpDNA) sequencing.

Miscanthus × *giganteus* is widely used in Europe for biomass production; its maximum yield reaches up to 45 t ha^{-1} yr^{-1} under irrigated conditions with low inputs due to the high water-, radiation-, and nitrogen-use efficiencies and nitrogen recycling (Zub and Brancourt-Hulmel, 2010). *Miscanthus* × *giganteus* displays a narrow genetic diversity among 15 clones from European collections (De Cesare et al., 2010). Therefore, breeding methods are under development to collect and improve the *Miscanthus* germplasm, and the determination of the genetic variation within and across *Miscanthus* species is useful for breeding new varieties at the intraspecific or interspecific level.

The cpDNA genome is maternally inherited and generally lacks recombination, making it a particularly relevant tool to investigate genetic variation, reveal the maternal parent of hybrid plants (Chiu and Sears, 1985), and survey phylogeographical patterns in plants (Su et al., 2004). Such coding plastid genes as *rbcL* and *matK* are largely used in phylogenetic studies; however, the level of conservation in the *rbcL* gene can be too high to clarify the relationships between very closely related genera (Gielly and Taberlet, 1994). Therefore, noncoding intron and intergenic spacers (IGSs) have proven to be more powerful tools because these regions should have a higher rate of variability than coding regions. Indeed, such IGSs as *trnL-F* have a mutation rate that is an average of more than three times that of the *rbcL* gene (Gielly and Taberlet, 1994). Molecular markers based on noncoding plastid regions have revealed polymorphisms, including simple sequence repeats (Powell et al. 1995a, 1995b; Provan et al., 2001), single nucleotide polymorphisms (SNPs), and insertion–deletion polymorphisms (Palmer et al., 1988), allowing powerful discrimination, even between closely related taxa (Provan et al., 2001; Flannery et al., 2006; McGrath et al., 2007). However, the availability of suitable cpDNA markers for *Miscanthus* remains limited in number: only 22 chloroplast microsatellite markers (cpSSR) have been developed to date (De Cesare et al., 2010; Jiang et al., 2012). Moreover, Al-Janabi et al. (1994) studied two other chloroplast intergenic spacer regions ($tRNA^{val}$–16S rRNA; rbcL–atpB) for the phylogenetics of sugarcane (*Saccharum officinarum* L.) and its relatives including *Miscanthus*. Four other chloroplast regions (*psbC-trnS*, *trnS-trnT*, *trnL-trnF*, and *rpl20-rps12*) were used to identify the putative maternal parent species of *Miscanthus* × *giganteus* (Hodkinson et al., 2002c; Dwiyanti et al., 2013) and *M. purpurascens* Andersson (Jiang et al., 2013).

The main objectives of the present study were (i) to investigate the genetic diversity of some natural *Miscanthus* accessions in China based on the frequency spectrum and geographic distribution of cpDNA haplotypes (i.e., combinations of alleles at adjacent loci on the chromosome, which are transmitted together), and (ii) to identify the Chinese genomes related to the maternal genomes of varieties cultivated in Europe for industrial or horticultural purposes. For these objectives, we predicted being able to identify enough polymorphisms from cpDNA between the species under investigation. We used sugarcane cpDNA due to its taxonomic relationship with *Miscanthus* and used primers based on the large single-copy region of the sugarcane chloroplast genome to describe the frequency spectrum of cpDNA haplotypes and their geographic distribution in China among 44 natural Chinese accessions native from 10 Chinese provinces. This also allowed the identification of the Chinese genomes related to the maternal progenitors of 21 European cultivated clones.

MATERIALS AND METHODS

Population Sampling

A total of 65 individuals were studied (Tables 1 and 2), including 44 Chinese accessions that corresponded to wild plants collected from diverse Chinese locations and 21 clones that are mostly cultivated in Europe for industrial or horticultural purposes. The material covered four species of *Miscanthus* s.l.

The 44 accessions of *Miscanthus* s.l. species were sampled from 10 provinces in central, southern, and eastern China: Anhui, Guangxi, Guangzhou, Shandong, Shanghai, Shǎnxi, Shānxi, Sichuan, Guizhou, and Zhejiang (Table 1). The accessions consisted of three species of *Miscanthus* [17 *M. sinensis*, 4 *M. sacchariflorus*, and 23 *M. floridulus* (Labill.) Warb. ex K. Schum. & Lauterb.] clones (Table 1). The plants collected in the field were propagated from rhizomes and were established in a nursery at a density of 2 plants m^{-2} in Linan Zhejiang Province (119°72′ N, 30°23′ E).

Using the morphological traits of the taxonomy revised by Sun et al. (2010), we classified individuals into separate species according to the flower characteristics for those individuals that could flower and according to such characters as rhizome development, stamen number, culm length, panicle size, and spikelet

Table 1. Location and ploidy level of 44 accessions of *Miscanthus* s.l. collected in China.

No.	Accession	Province	Latitude	Longitude	Species	Ploidy level
			°E	°N		
1	N-1	Zhejiang	28.90	118.50	*M. floridulus*	2*x*
2	N-2	Zhejiang	28.64	121.27	*M. floridulus*	2*x*
3	N-4	Zhejiang	29.15	118.39	*M. floridulus*	2*x*
4	N-6	Zhejiang	30.16	118.36	*M. floridulus*	2*x*
5	N-7	Zhejiang	29.11	118.20	*M. floridulus*	2*x*
6	N-8	Zhejiang	29.13	118.54	*M. floridulus*	2*x*
7	N-9	Zhejiang	29.00	119.10	*M. floridulus*	2*x*
8	N-10	Zhejiang	28.22	121.21	*M. floridulus*	2*x*
9	N-11	Zhejiang	30.43	120.30	*M. floridulus*	2*x*
10	N-12	Zhejiang	30.43	120.30	*M. sinensis*	2*x*
11	N-19	Zhejiang	29.32	121.30	*M. sinensis*	2*x*
12	N-20	Shănxi	34.17	108.57	*M. sinensis*	2*x*
13	N-21	Shanghai	31.10	121.40	*M. floridulus*	2*x*
14	N-23	Zhejiang	30.07	119.25	*M. sinensis*	2*x*
15	N-24	Zhejiang	28.27	119.54	*M. floridulus*	2*x*
16	N-25	Guangzhou	23.04	113.31	*M. sinensis*	2*x*
17	N-27	Zhejiang	28.74	118.61	*M. floridulus*	2*x*
18	N-29	Zhejiang	28.27	119.54	*M. floridulus*	2*x*
19	N-32	Zhejiang	27.48	120.38	*M. sinensis*	2*x*
20	N-33	Zhejiang	27.48	120.38	*M. floridulus*	2*x*
21	N-34	Anhui	31.67	115.87	*M. floridulus*	2*x*
22	N-35	Shandong	36.65	117.00	*M. sinensis*	2*x*
23	N-36	Zhejiang	28.08	119.18	*M. floridulus*	2*x*
24	N-37	Zhejiang	28.08	119.18	*M. sinensis*	2*x*
25	N-111	Zhejiang	29.49	119.27	*M. floridulus*	2*x*
26	N-112	Zhejiang	29.49	119.27	*M. floridulus*	2*x*
27	N-113	Sichuan	30.42	103.73	*M. sinensis*	2*x*
28	N-116	Shănxi	34.18	108.97	*M. floridulus*	2*x*
29	N-117	Shānxi	36.29	111.90	*M. sacchariflorus*	2*x*
30	N-119	Guangxi	24.15	115.75	*M. floridulus*	2*x*
31	N-120	Guangxi	23.17	108.27	*M. floridulus*	2*x*
32	N-122	Guangxi	24.15	115.75	*M. floridulus*	2*x*
33	N-125	Shānxi	36.08	111.50	*M. sacchariflorus*	2*x*
34	N-128	Shānxi	36.08	111.50	*M. sacchariflorus*	2*x*
35	N-131	Sichuan	34.18	108.97	*M. floridulus*	2*x*
36	N-132	Shănxi	34.38	109.22	*M. sacchariflorus*	2*x*
37	N-134	Guangzhou	23.16	113.23	*M. sinensis*	2*x*
38	N-136	Guangzhou	23.17	113.22	*M. sinensis*	2*x*
39	N-137	Guangzhou	28.82	120.12	*M. sinensis*	2*x*
40	N-138	Guangzhou	23.17	113.22	*M. sinensis*	2*x*
41	N-139	Guangzhou	23.17	114.38	*M. sinensis*	2*x*
42	N-140	Guizhou	26.74	109.08	*M. sinensis*	2*x*
43	N-141	Guangzhou	23.11	113.11	*M. sinensis*	2*x*
44	N-142	Zhejiang	29.56	119.78	*M. sinensis*	2*x*

shape (Hodkinson et al., 1997; Sun et al., 2010; Chen and Renvoize, 2006) (Table 1) for all individuals. *Miscanthus sacchariflorus* can be readily distinguished from the other species by morphological characters, such as its elongated or creeping rhizome, lack of awns, and callus hairs, which are more than twice the spikelet length. *Miscanthus sinensis* is a widely distributed species

Table 2. Description of the 21 European *Miscanthus* clones, including species, ploidy level, code, name, and provider (adapted from Zub et al., 2012).

Species	Ploidy level	Code	Name	Acquired from
M. floridulus†	3*x*	Flo	*M.* × *giganteus* 'Floridulus'	Chombart, France
M. × *giganteus* (interspecific hybrid)	3*x*	GigB	*M.* × *giganteus* UK	ADAS‡
	4*x*	GigD	*M.* × *giganteus* DK	Nordic biomass
M. sacchariflorus × *M. sinensis*	2*x*	H8§	*M. sacchariflorus* × *M. sinensis*	Danish institute of agricultural science, Aarhus
M. sinensis	2*x*	Aug	*M. sinensis* 'August Feder'	Chombart, France
	2*x*	Fla	*M. sinensis* 'Flamingo'	Chombart, France
	2*x*	Grz	*M. sinensis* 'Graziella'	Chombart, France
	2*x*	Mal	*M. sinensis* 'Malepartus'	Chombart, France
	2*x*	Rot	*M. sinensis* 'Rotsilber'	Chombart, France
	2*x*	Sil	*M. sinensis* 'Silberspinne'	Chombart, France
	2*x*	Str	*M. sinensis* 'Strictus'	Chombart, France
	2*x*	Fer	*M. sinensis* 'Ferner Osten'	Bruckeveld, Belgium
	2*x*	Her	*M. sinensis* 'Herman Müssel'	Bruckeveld, Belgium
	2*x*	Punk	*M. sinensis* 'Punktchen'	Bruckeveld, Belgium
	2*x*	Pur	*M. sinensis* 'Purpurescence'	Bruckeveld, Belgium
	2*x*	Yak	*M. sinensis* 'Yaku Jima'	Bruckeveld, Belgium
	4*x*	Gol	*M. sinensis* 'Goliath'	Chombart, France
	4*x*	GolD	*M. sinensis* 'Goliath Danois'	Nordic biomass
	3*x*	H6§	*M. sinensis* Hybrid	Danish institute of agricultural science, Aarhus
M. sacchariflorus	4*x*	H5§	*M. sacchariflorus* Hybrid	Danish institute of agricultural science, Aarhus
	2*x*	Sac	*M. sacchariflorus*	Chombart, France

† Sold as a clone of the *M. floridulus* species, but Zub et al. (2012) showed that the correct species is *M.* × *giganteus*.

‡ Agriculture Development and Advisory Service.

§ Novel clones bred at Aarhus University (DK) and acquired from Uffe Jorgensen.

with a broad variation in foliage and spikelet color. However, it is very similar morphologically to *M. floridulus*, and their distributions largely overlap. These two species mostly differ with regard to the relative length of the axis and panicle, with the axis-to-panicle length ratio being over 2:3 for *M. floridulus* but between 1:5 and 1:2 for *M. sinensis*.

In addition, 21 clones were assembled in a nursery in the Institut National de la Recherche Agronomique (INRA) unit at Estrées-Mons (49°53′ N, 3°00′ E) in France (Table 2): 18 clones corresponded to varieties, and 3 clones corresponded to breeding material from the Danish University of Aarhus (H5, H6, and H8) (for further description, see Clifton-Brown et al. [2001] and Zub et al. [2011]). Using the taxonomic key of Sun

et al. (2010) and with the exception of the cultivar Flo, all the *M. sacchariflorus* and *M. sinensis* accessions were accurately classified. Although Flo was obtained from an ornamental flower company that sold it as a clone of *M. floridulus*, the validity of this assignment is doubtful; thus, the species level was not identified in the present study (Zub et al., 2012). Three *Miscanthus* spp. hybrids (H5, H6, and H8) were acquired from U. Jorgensen of the Danish University of Aarhus. Two clones of *M.* × *giganteus* were provided by the Agriculture Development and Advisory Service (ADAS) and the Danish University of Aarhus (Table 2). More detailed information regarding the cultivated clones in France can be found in Zub et al. (2011, 2012).

Determination of the Ploidy Level

As the ploidy level was not previously available for the Chinese accessions, it was evaluated using flow cytometry. Fully expanded leaves were collected from the Linan nursery-grown plants, and the material was washed in distilled water. To isolate and stain nuclei, young leaves were chopped into 5-mm^2 discs for 5 min using a razor blade in a plastic petri dish containing 400 μL of extraction buffer (Partec CyStain PI Absolute P Nuclei Extraction Buffer, Partec GMBH, Munster, Germany). After adding 1.6 μL of staining buffer (Partec CyStain PI Absolute P Staining Buffer, Partec GMBH, Munster, Germany), the material was incubated for 10 min. The measurements were performed with a 10-min period using a CyFlow Ploidy Analyser (Partec) using a 488-nm laser light source. We compared a histogram of the fluorescence intensities of the given sample with a reference of plants with known ploidy levels. We used two references: triploid *M.* × *giganteus*, acquired from Bruckeveld, Belgium, which corresponded to the clone from ADAS, and diploid *M. sinensis* Ferner Osten from Bruckeveld, Belgium. The ploidy of the references were determined by Zub et al. (2012) by flow cytometry and using H5 which was tetraploid (Clifton-Brown et al., 2001) and the triploid *M.* × *giganteus* from ADAS as references.

The ploidy level of the 21 clones established in the INRA unit at Estrées-Mons, France, was described by Zub et al. (2012).

Identification of Chloroplast DNA Variation

DNA Extraction and Amplification

Fresh leaf tissue was sampled in the field for each individual in the Chinese and French nurseries. The Chinese samples were dried in silica gel and transported to the laboratory for DNA analyses. Total genomic DNA was isolated from the dry specimens using the cetyl trimethyl ammonium bromide method (Hodkinson et al., 2002a).

The French samples were stored in the laboratory at −80°C. The frozen leaves were ground into a powder in liquid nitrogen. The total genomic DNA of the French samples was extracted using the NucleoSpin Plant II kit (Macherey Nagel, Germany), with some adjustments. We followed the protocol of the NucleoSpin Plant II kit, except that we incubated the suspension for 30 min at 65°C during the cell lysis using Buffer PL1. When eluting the highly pure DNA, we pipetted 70 μL Buffer PE (70°C) onto the membrane and incubated for 5 min at room temperature before centrifugation. We repeated this step two more times.

For the polymerase chain reaction (PCR) amplification, 30 ng template genomic DNA was combined in a 25-μL volume containing 10× PCR buffer (Qiagen, USA), 1.5 or 3 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 μM each primer, and 0.5 units *Taq* DNA polymerase (Qiagen). The PCR amplification was performed using an Eppendorf Authorized Thermal Cycler with the following procedure: an initial denaturation step of 2 min at 95°C, followed by seven cycles using a touch-down protocol (45 sec at 94°C, 45 sec of annealing starting from 62°C to 56°C, with a decrease of 1 degree every cycle, and 1 min of elongation at 72°C), then 30 cycles (45 sec at 94°C, 45 sec of annealing at 55°C, and 1 min at 72°C), and a final extension at 72°C for 5 min. The final PCR products were evaluated on 1% agarose gels stained with ethidium bromide and then sequenced by GenoScreen (Lille, France).

Design of Chloroplast DNA Primer Pairs and Selection for Sequencing

To design the new markers, we referred to Grivet et al. (2001), who used the chloroplast genome from tobacco (*Nicotiana tabacum* L.) as a reference to design 38 primer pairs, demonstrating a good conservation of those primer pairs for most eudicots. We decided to design these markers on the large single-copy region of the sugarcane chloroplast genome (DDBJ/EMBL/GenBank accession number NC_006084.1) due to the close taxonomic relationship between this plant and *Miscanthus*. Forty-five new cpDNA primer pairs were designed according to thermodynamic parameters using Primer3 (V.0.4.) (http://frodo.wi.mit.edu/ [accessed 20 Jan. 2014]) (Table 3 and Supplemental Table S3). The primers based on the coding sequences of two close genes were designed to amplify as much as possible of the noncoding sequences. We also decided on a cutoff of approximately 900 bp as the length of each PCR product for consistency with the sequencing constraints.

Before the amplification, a preliminary screening of the primer pairs was performed using a sample of 10 individuals randomly chosen from the four species of *Miscanthus*. We retained only the primer pairs for which we obtained a single band of the expected size calculated on sugarcane. After sequencing the retained PCR products, polymorphisms within the sample pool were detected for 20 cpDNA markers (KS, CZ, GO81, O81O147, TE, ED, MN, S2I, AS14, AAY3, Y3S3, S3S4, FHJ, VE, AIY4, JE, BN, NTB, TDA, and S3S19a) (Table 3). Each marker locus varied in its ability to differentiate the chloroplast genome types and taxa (Supplemental Table S1). The S3S4 sequence was compared to the *trnS-trnT* sequence of Dwiyanti et al. (2013) with which it shared 842 bp (Supplemental Table S2).

Then five primer pairs (AAY3, VE, BN, NTB, and TDA), which covered all types of genetic polymorphisms found for the 10 individuals, were chosen to amplify all 65 individuals. These primer pairs resulted in the amplification of a product from all individuals of all four species.

Data Analysis of Chloroplast DNA Sequence Variation

The forward and reverse sequence data were analyzed and aligned using the program Genious pro 4.8.3 (www.geneious.com [accessed 20 Jan. 2014]). All insertion-deletions (corresponding to mutation classes) were coded and treated as single point mutations. Poly-A and poly-T variations were excluded from the analyses because of the uncertainty related to homoplasy, that is, similarity due to convergent evolution but of independent origin. The cpDNA haplotypes were determined

Table 3. Primer sequence description and location of 20 chloroplast DNA primer pairs that were found to be polymorphic among the four species of *Miscanthus*. Marker names in bold correspond to the five markers we chose to amplify all 65 individuals.

No.	Marker name	Location in sugarcane	Forward primer	Sequence	Reverse primer	Sequence	PCR† product size
							bp
1	KS	7550–8448	psbK-F	ATTTTCAACCCAATCGTGGA	trnS-R	GGAGAGATGGCTGAGTGGAC	807
2	CZ	10434–12629	psbC-F	GGTTAGCGACCTCCCATTTT	psbZ-R	GACCAACCATCAGGAGAAGC	836
3	GO81	13649–15201	trnG-F	TTGGATACTAATCGCGAGAATG	orf81-R	ATGGAGGGGATCGACTAACC	862
4	O81O147	14956–15932	orf81-F	GGTTAGTCGATCCCCTCCAT	orf147-R	GGATACAACAATGGGCCAAA	848
5	TE	16373–17051	trnT-F	GGTAGAGTAATGCCATGGTAAGG	trnE-R	CCCAGGGGAAGTCGAATC	660
6	ED	16979–17619	trnE-F	CCCCATCGTCTAGTGGTTCA	trnD-R	CAGCTTCCGCCTTGACAG	616
7	MN	18671–19675	psbM-F	AAAAACAGCCAGTCAAAATGA	petN-R	AGTATGGGGGAGGAGTGGAC	861
8	S2I	32355–34059	rps2-F	TTCCACCATCTCCCAAAAAG	atpI-R	GGTTTGTGGATTCCGAACAG	764
9	AS14	37078–39528	atpA-F	CCAGTGGAAGAGCAGGTAGC	rps14-R	GTTACCGGGTGCAACAAGAT	884
10	**AAY3**	41903–46769	psaA-F	GGGGCCCTTAGCTATTGTTC	ycf3-R	TTGGTTTGATCAAGCTGCTG	837
11	Y3S3	44760–47444	ycf3-F	TGCGAACCCTCTCTCTTTCT	trnS3-R	CGAACCCTCGGTAAACAAAA	853
12	S3S4	47358–48330	trnS3-F	TTTTGTTTACCGAGGGTTCG	rps4-R	AAAACGCCTAAATCCGGAAG	854
13	FHJ	50425–51546	trnF-F	CCTCGTGTCACCAGTTCAAA	ndhJ-R	GAATCCCGTCTGTTTTCTGG	795
14	**VE**	53673–55165	trnV-F	TTTTTGGAGGCCCTTATCCT	atpE-R	GCACTTGAAATAGCCGAAGC	824
15	AIY4	59830–60858	psaI-F	GGCAATTGCAATGACTTCCT	ycf4-R	GATTGGATATCCCGCACAAG	796
16	JE	64013–64781	psbJ-F	CCCAATCCAGAATACGAACC	psbE-R	GTCTGGAAGCACGGGAGA	755
17	**BN**	71341–73341	psbB-F	TAGGCGTAACGGTGGAGTTC	psbN-R	CGCCATCTCCATATCTGGTT	799
18	**NTB**	73210–75200	psbN-F	GCCCAAACGCGGTATATAAG	petB-R	TTTTGGGCAAACAAAATTGG	798
19	**TDA**	75383–77845	petD-F	ATCCGTTTGCAACTCCTTTG	rpoA-R	GCGCTTTATGAAGCTTCTCG	886
20	S3S19a	81811–83365	rps3-F	GGAATGGTGATTTTGGGTTG	rps19a-R	GGCAAAAATCGAAAAGGTCA	897

† PCR, polymerase chain reaction.

based on the aligned sequences (the haplotypes were arbitrary coded from A to J), and a statistical parsimony haplotype network was constructed using the program TCS 1.2.1 (Clement et al., 2000) with the method described by Templeton et al. (1992).

We used the sequence query to describe the genetic distance between each individual. Using the platform "phylogeny.fr" (Dereeper et al., 2008), a consensus phylogenetic tree reconstruction was obtained from the set of sequences by bootstrapping, and the robustness of the topology was determined using 1000 bootstrap replicates. The phylogenetic tree was constructed with the PhyML 3.0 program of the platform, which, by default, estimates the parameters of a HKY85 substitution model (Hasegawa et al., 1985); it simulates how one sequence of nucleotides may have evolved from another, as nucleotides within a sequence can evolve via substitution under the assumption that the rates of substitution differ between each nucleotide. This program combines information from several loci to cluster individuals within a predefined number of groups: we used the advanced settings, which allowed a γ distribution parameter across four categories of nucleotide sites and a transition/transversion ratio fixed at 4.

RESULTS

Phylogenic Relationships and Diversity of Chloroplast Haplotypes

We developed 45 new cpDNA primer pairs using the cpDNA sequence of sugarcane as a reference. A total of 35 of these primer pairs (77.8%) successfully amplified a product for all species of *Miscanthus* s.l. Among the 45 markers, 20 (44.4%) were polymorphic (Table 3 and Supplemental Table S3).

DNA sequences were determined for five markers (AAY3, VE, BN, NTB, and TDA) (Table 3), covering most of the variation observed between the four species of *Miscanthus*, as based on a preliminary sample of 10 individuals (as shown by Supplemental Table S1). These five new chloroplast markers were then tested with 21 cultivated clones and 44 natural accessions. High levels of polymorphisms were detected: 17 were single-site mutations, 3 were sequence repeat variations (3–64 bp in length) (Table 4), and 3 were mononucleotide repeat poly-T and poly-A sites (data not shown). One-third of these polymorphisms corresponded to coding regions, whereas two-thirds corresponded to noncoding regions (data not shown). Among these polymorphisms, six were detected in the *psaA-ycf3* region (AAY3), followed by four in the *psbB-psbN* region and four in the *psbN-petB* region (BN and NTB), three in the *petD-rpoA* region (TDA), and three in the *trnV-atpE* region (VE). The markers did not overlap, with the exception of BN and NTB at 65 bp. We did not retain common polymorphisms to avoid counting them twice.

Altogether, based on the 20 polymorphisms detected (except the repeat poly-A and poly-T sites), the sequences obtained distinguished 10 haplotypes (A to J in Table 4). The phylogenetic relationships among the haplotypes were inferred by statistical parsimony and were depicted as a network based on mutational steps (Fig. 1 and see Fig. 2, which displays the individuals for each haplotype). Except for three cases (I–H, H–J, and C–D), the haplotypes were generally separated by more than one mutation.

Table 4. Description of 10 haplotypes (A to J) identified by the five markers (AAY3, VE, BN, NTB, and TDA) of *Miscanthus* s.l. selected in Table 3. All sequences are in comparison to the reference haplotype A. A dot indicates that the same nucleotide as for haplotype A is present. The numbers (1× or 2×) correspond to the number of the repeated pattern (28-bp, 64-bp, or 3-bp)†.

Marker	Nucleotide position	Haplotype									
		A	B	C	D	E	F	G	H	I	J
AAY3	107	C	.	T	T	T	T	T	T	T	T
AAY3	149	T	.	.	.	.	.	.	G	G	G
AAY3	428	1× 28-bp	2× 28-bp	.	.	.	.	.	.	.	.
AAY3	466	C	.	.	.	.	.	A	.	.	.
AAY3	598	T	G	G	G	G	G	G	G	G	G
AAY3	640	G	A	.	.	.	.	.	.	.	.
VE	102	G	.	A	A	A	A	A	A	A	A
VE	203	G	.	.	.	A	.	.	.	.	.
VE	365	C	T	.	.	.	.	.	.	.	.
BN	316	C	.	.	.	.	.	T	.	.	.
BN	358	1× 64-bp	.	.	.	2× 64-bp	.	.	.	.	.
BN	529	G	.	.	.	.	.	.	.	A	.
BN	587	G	.	.	.	.	T	T	T	T	T
NTB	145	G	.	.	.	.	T	.	.	.	.
NTB	389	C	.	T	T	T	T	T	T	T	T
NTB	417	C	T	T	T	T	T	T	T	T	T
NTB	609	T	.	.	.	.	.	.	.	.	C
TDA	290	2× 3-bp	.	1× 3-bp	1× 3-bp	1× 3-bp	1× 3-bp	1× 3-bp	1× 3-bp	1× 3-bp	1× 3-bp
TDA	380	G	.	.	.	.	T	.	.	.	.
TDA	743	A	.	G	.	.	.	.	.	.	.

† 28-bp = TCCTTGAGTGAGCATAATATAGATTTTT; 64-bp = TTTGATTTGACATGAGGAACATCTCCTGTCCTTTCTTTGACTCTTTTTCTTTTTTTATATGGGA; 3-bp = AAG.

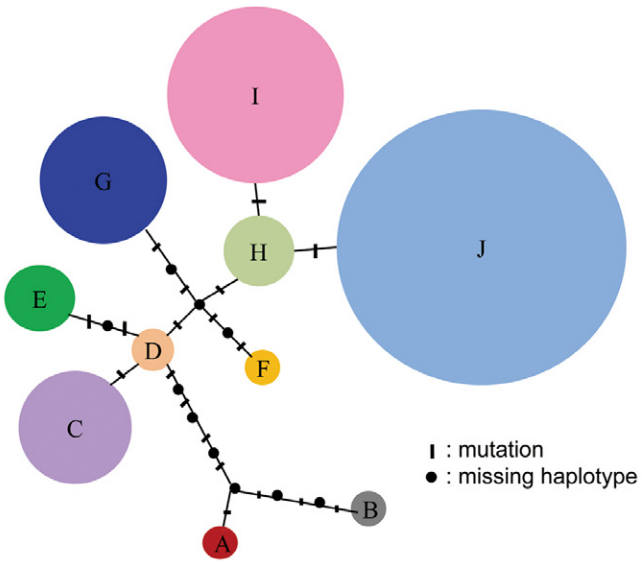

Figure 1. Phylogenetic reconstruction of the relationships between haplotypes (A to J) in *Miscanthus* s.l. using the HKY85 substitution model. The 10 haplotypes are described in Table 4 and the individuals are detailed in Fig. 2 for each haplotype. The size of the pies is proportional to the number of individuals carrying the corresponding haplotype.

Haplotypes I and J appeared to be derived from haplotype H. Haplotypes C and E were quite close to D and might have originated from haplotype D because haplotype D had the highest number of connections.

Haplotypes A and B were also separated from each other by more than five mutational steps. These two haplotypes were more distant from the others.

Based on the phylogenetic tree reconstruction of the cpDNA haplotype relationships, the frequency of the haplotypes within the haplotype network varied. Haplotype J was the most frequent, being found in 29 individuals (44.6% of all samples). Haplotypes I and G were less represented, with 15.4% and 23.0% of the total individuals, respectively.

Identification of the Maternal Relationships between the European Cultivars and Wild Accessions in China

The phylogenetic tree based on a matrix of genetic distances to study the maternal relationships between cultivated and wild accessions revealed several main features (Fig. 2).

First, two wild accessions of *M. sinensis* and *M. floridulus*, group A and B, constituted a clade and formed a deep phylogenetic split from the others. Second, the tetraploid clone of *sacchariflorus* (H5), which possesses haplotype D, had a high genetic similarity value with group C, which contains clones of *M.* × *giganteus* (0.79), and group E, comprising two clones of *M. sacchariflorus* (0.94), but had no common maternal genome with any wild accession we

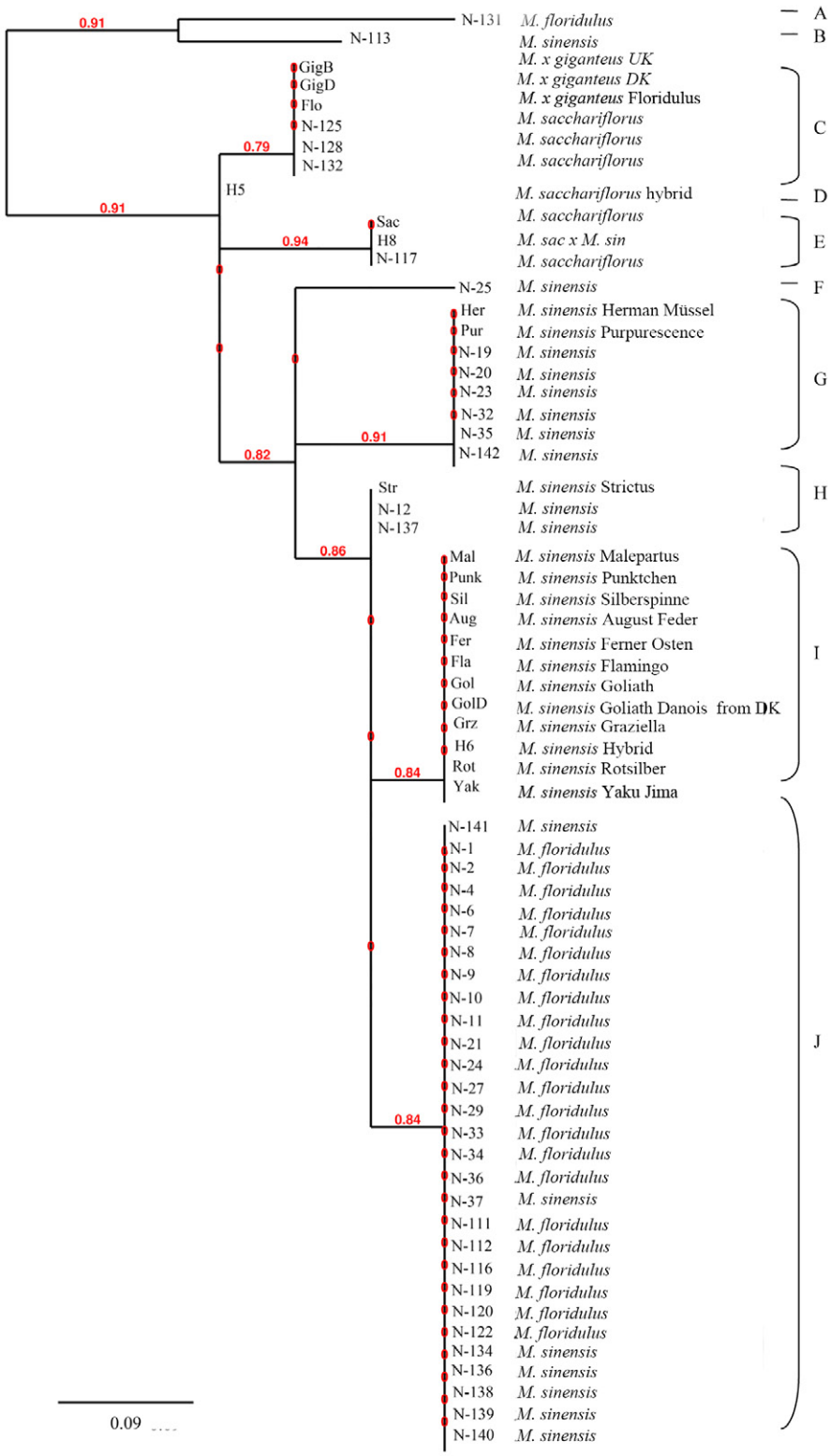

Figure 2. Phylogenetic tree showing the relationships between *Miscanthus* s.l. individuals, as revealed by chloroplast DNA markers. Species names and coding of each individual are indicated as in Tables 1 and 2. The Ncoding refers to the wild Chinese individuals. The letters (A to J) correspond to haplotypes described in Table 4. The numbers above the nodes represent the genetic similarity value.

collected. *Miscanthus sacchariflorus* (Sac) and a hybrid (H8) between *M. sinensis* as male parent and *M. sacchariflorus* as maternal parent shared the same maternal genome (haplotype E) with a wild *M. sacchariflorus* (N-117). The two clones of *M.* × *giganteus* (GigB and GigD) were grouped with three wild *M. sacchariflorus* accessions (N-125, N-128, and N-132), thus revealing the same maternal genome (haplotype C). Group C, which contained all the cultivated *M.* × *giganteus*, had no maternal genome related to the clone of *M. sacchariflorus* (Sac) cultivated in Europe.

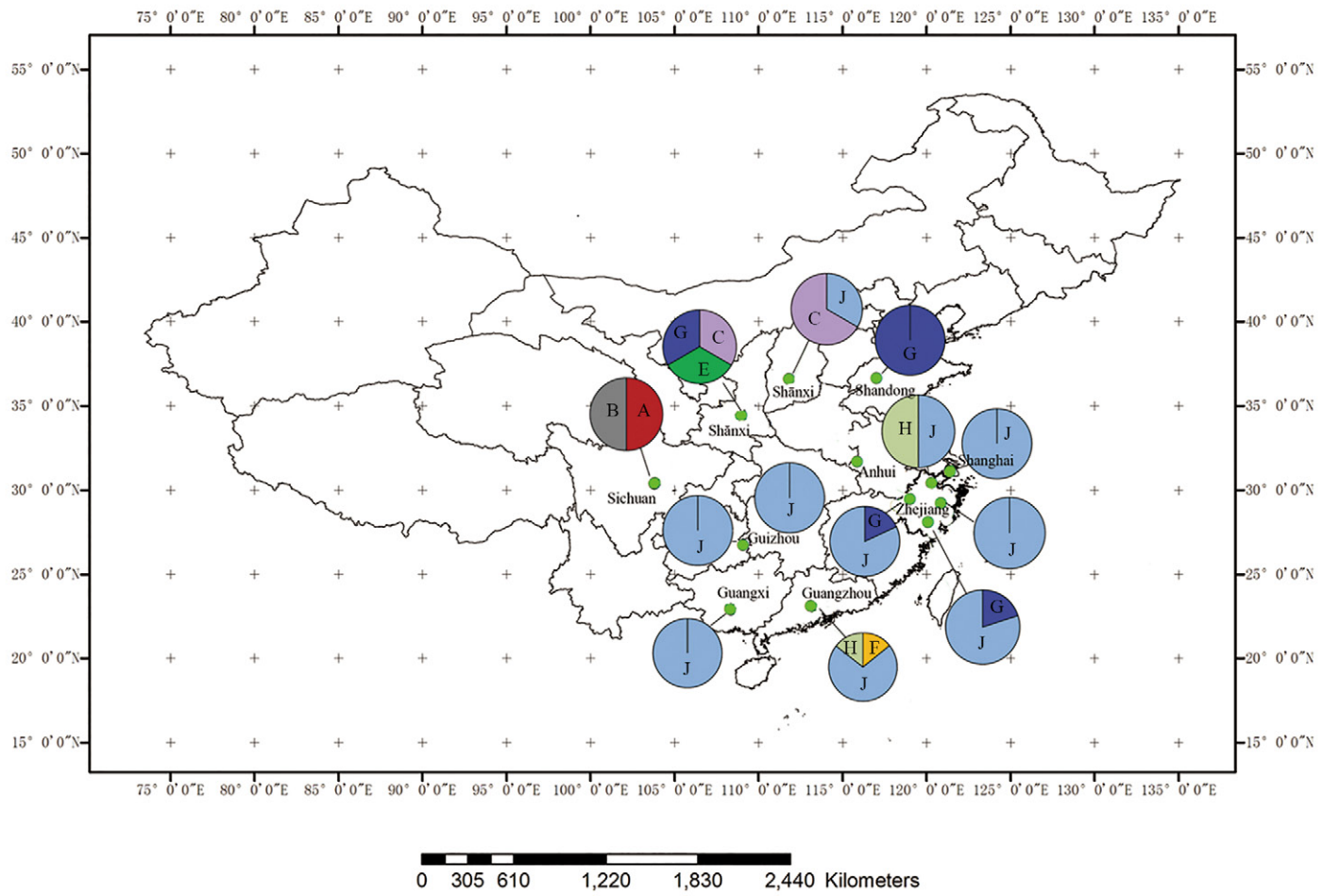

Figure 3. The geographic distribution of chloroplast DNA haplotypes in China, as indicated using the same circle colors as in Fig. 1. The accessions correspond to those detailed in Table 1. The letters A to J correspond to the haplotypes detailed in Fig. 2.

Although it is likely that the species *M. sacchariflorus* was the maternal parent of the *M.* × *giganteus* available in Europe, our data indicate that this cultivated *M. sacchariflorus* clone (Sac) is not the maternal parent of the *M.* × *giganteus* clones studied here. Interestingly, the cultivar *M. floridulus* (Flo) was grouped together with *M.* × *giganteus* from group C and was clearly separated from the other *M. floridulus* wild accessions.

Finally, the tree indicated that the remaining clones were separated into five closely related but distinct groups. Low contrasting levels of intraspecific variations were discovered among the cultivars of *M. sinensis*. Her and Pur had the same maternal pedigree as the wild accessions N-19, N-20, N-23, N-32, N-35, and N-142 and shared the same G haplotype. Str possessed the same maternal genetic background as the wild accessions N-12 and N-137. However, the remaining *M. sinensis* genotypes, Mal, Punk, Sil, Aug, Fer, Fla, Gol, GolD, Grz, H6, Rot, and Yak, possessed haplotype I, which did not group with any of the wild accessions we sampled. Haplotype J appeared to be specific to the wild accessions, as it only occurred among the accessions found in China (Fig. 2).

Geographic Distribution of the Chloroplast Haplotypes from Wild Accessions in China

The geographic distribution of the chloroplast haplotypes in China was examined using the natural accessions indicated in Table 1.

For *Miscanthus*, haplotype J was the most frequent and was widely distributed all over central, southern, and eastern China (Fig. 3). Haplotype G was also distributed in many regions (Zhejiang, Shandong, and Shănxi). Haplotype H occurred in Zhejiang and Guangzhou provinces, whereas haplotypes C and E were found only in the adjacent Shănxi and Shānxi provinces. Haplotypes A and B, which were highly distant from the others in the haplotype network, were only found in the Sichuan Basin (Fig. 1 and 3). In particular, Guangzhou, Zhejiang, and Shănxi provinces harbored a number of haplotypes that could be used as new germplasm resources.

DISCUSSION

Although widely cultivated in Europe, *M.* × *giganteus* displays a narrow genetic diversity. Discovering new germplasm unrelated to the current varieties is therefore critical to enlarge the availability of *Miscanthus* varieties for plant

breeding. Among others, cpDNA can help breeders for searching such unrelated germplasm. We designed primers using a large single-copy chloroplast region from sugarcane. Using these primers, we were able to identify the genomic relationships between 21 clones, most of which are currently cultivated in Europe, and 44 Chinese wild accessions. It was then hypothesized that the origin of the cultivated clones could be determined if they shared a maternal genome with the wild accessions. Below, we discuss the power of new cpDNA markers for studying cpDNA diversity and studying the haplotype structure in *Miscanthus*. Second, we focus on the maternal genome of *M.* × *giganteus* and its relationship with the region of Chinese wild clones of *M. sacchariflorus* and *M. sinensis*. Lastly, we identified three Chinese provinces as potential genetic sources of *Miscanthus* s.l.

The Power of New Chloroplast DNA Markers for Chloroplast DNA Diversity and Haplotype Structure Studies in *Miscanthus*

The chloroplast gene order in angiosperms is conserved, there is a lack of recombination, and the maternal inheritance enables the elucidation of the relative contribution of maternal genetic structure from natural populations (Provan et al., 2001). Using six cpSSRs, De Cesare et al. (2010) differentiated *M. sinensis* from *M. sacchariflorus* and *M.* × *giganteus* but failed to separate *M. sacchariflorus* and *M.* × *giganteus*. Because there are many intergenic regions other than those traditionally used in plants that offer useful levels of variation, we sought to prospect a sequencing strategy of long chloroplast regions (mostly intergenic) rather than sequencing cpSSRs. This strategy allowed us to obtain a large number of suitable polymorphisms with only a few markers (20 polymorphisms were revealed for five markers).

In the preliminary research we conducted, most of the primer pairs of Grivet et al. (2001), designed for tobacco, failed to amplify *Miscanthus* DNA, providing an amplification rate of approximately 23%. This may have been because the orientation and relative positions of some genes in the chloroplast genome differed between *Nicotiana* and *Miscanthus*, as hypothesized earlier between *Nicotiana* and *Saccharum* (Calsa et al., 2004).

In the present study, new cpDNA markers using the large single-copy region of the sugarcane chloroplast sequence were developed for *Miscanthus*, which includes cultivars and wild accessions. Some 77.8% of the primers successfully generated products and were readily sequenced across the accessions of *Miscanthus* used in this study. Each of the 20 chloroplast regions (Table 3 and Supplemental Table S1) under investigation contained various sequence variations among the four species of *Miscanthus* (*M. sinensis*, *M.* × *giganteus*, *M. sacchariflorus*, and *M. floridulus*). More particularly, we found a high overall level of polymorphisms located in the regions of *psaA-ycf3*. Dwiyanti et al. (2013) investigated cpDNA regions of *psbC-trnS*, *trnS-trnT*, *trnL-trnF*, and *rpl20-rps12* to distinguish the species of *M. sinensis*, *M.* × *giganteus*, and *M. sacchariflorus*. Jiang et al. (2013) used the same *trnL-trnF* as Dwiyanti et al. (2013) to detect the maternal lineage of a natural diploid hybrid with its two parents, *M. sinensis* and *M. sacchariflorus*. Among 20 polymorphic primer pairs from the 45 designed (Table 3 and Supplemental Table S1), 19 pairs are novel in our study compared to the validated *Miscanthus* cpDNA markers (Dwiyanti et al., 2013; Jiang et al., 2013). These 19 sequences differed from those of Dwiyanti et al. (2013) because they were designed at distinct locations on the genes, which involved different amplified sequences. Regarding our 20th S3S4 sequence, it shared 842 bp with their *trnS-trnT* sequence and it distinguished the species in agreement with their results (Supplemental Table S2). In conclusion, the newly developed cpDNA primers can increase the availability of useful tools for studying the maternally inherited genomes of *Miscanthus*. For breeding purposes, it could be helpful, for instance, for the elucidation of the origins of the cultivars.

Genetic Comparison of Wild Chinese Accessions and European Cultivated Species

The haplotype variation of the available cultivars in our material was low (Fig. 2). These cultivars corresponded to the European cultivated species for which the conscious domestication of the plants and trait selection could explain this low variation (Provan et al., 2001). In a comparison between material from Japan and the United States, the genetic structure was also found to be higher in the Japanese populations than in the introduced populations in United States where some species are widely commercialized (Quinn et al., 2012).

Most members of *Miscanthus* s.l. are endemic to Asia (China, Japan, and neighboring regions) and Pacific islands (Hodkinson et al., 2002a; Clifton-Brown et al., 2008). Although three putative triploid hybrids were discovered in a sympatric population of tetraploid *M. sacchariflorus* and diploid *M. sinensis* by Dwiyanti et al. (2013) in Japan, we have not found any natural triploid in the Chinese provinces we prospected to date. However, three *M.* × *giganteus* accessions possessed the same C haplotype as the wild accessions of *M. sacchariflorus* collected in the Shānxi and Shǎnxi provinces of China, and cultivar *M. sacchariflorus* had the same E haplotype as the wild relative found in Shānxi Province. It is in agreement with Hodkinson et al. (2002c), who showed that the maternal lineage of *M.* × *giganteus* was *M. sacchariflorus* using cpDNA. In addition, although *M.* × *giganteus* shared DNA sequences with wild Chinese *M. sacchariflorus* clones, our data show it is not related to the *M. sacchariflorus* clone found in Europe for ornamental use.

In conclusion, our results show that wild Chinese *M. sacchariflorus* are related to the female parent of the *M.* × *giganteus* clones cultivated in Europe.

Three Chinese Provinces Are Identified as Potential Genetic Sources of *Miscanthus* s.l.

The widespread distribution of haplotype J related to *M. floridulus* overlaps with the G and H haplotypes belonging to *M. sinensis* (Fig. 3). Hodkinson et al. (2002b) indicates that *M. floridulus* is generally tropical Pacific in distribution and overlaps with *M. sinensis*. Nevertheless, according to Chen and Renvoize (2006), this species is also wildly adapted to different habitats from slopes, valleys, and grasslands. These two closely related species appear to intergrade: they gradually merged with one another through a continuous series of intermediate forms, and they overlap in their distribution. Sun et al. (2010) found that there are overlaps between *M. floridulus* and *M. sinensis*, particularly for those naturally occurring in Guangdong, Guangxi, Guizhou, Hunan, Sichuan, and Yunnan. Intermediate types for the morphological traits studied were also found, and according to the authors, a genetic study of these intermediate types needs to be conducted to clarify whether they are natural interspecific hybrids.

Although diversity in our study was most likely lower than the diversity covered by Sun et al. (2010), owing to the large number of accessions they studied, we obtained a clear distinction between the maternal genomes of *M. sinensis* and *M. floridulus*. We even found a high diversity of haplotypes in Shănxi, Guangzhou, and Zhejiang provinces, which suggests that these provinces are a potential source of endemic genetic material (Fig. 3 and Table 1) and that natural selection of the germplasm in these habitats may enlarge the genetic pool, assuming the maternal parent reflects the genetic diversity.

As the genetic variability of *M.* × *giganteus* is known to be narrow (Hodkinson et al., 2002c), the accessions from these provinces could provide interesting new genetic variability to enlarge the pool of available *M.* × *giganteus* varieties.

CONCLUSION

Our study defined for the first time the relationships between the maternal genome in most cultivars of *Miscanthus* grown in Europe and accessions from China. The newly designed cpDNA primers allowed the discovery of natural *Miscanthus sacchariflorus* accessions in China that are related to *M.* × *giganteus*. This increases the availability of molecular tools for identifying the maternal lineage of cultivated *Miscanthus* and studying diversity in plant evolution.

Most of the mutations detected by the cpDNA primers we used were substitutions. These new SNPs could be useful to breeders to detect interesting germplasm among diverse collections.

Supplemental Information Available

Supplemental information is included with this article.

Supplemental Table S1. Description of nucleotides of 20 polymorphic markers for 10 individuals among *Miscanthus sinensis*, *M. sacchariflorus*, *M.* × *giganteus*, and *M. floridulus*.

Supplemental Table S2. Comparison of the common sequence *trnS-trnT* with the sequence published by Dwiyanti et al. (2013). Results acquired for eight individuals among *Miscanthus sinensis*, *M. sacchariflorus*, *M.* × *giganteus*, and *M. floridulus*.

Supplemental Table S3. Primer sequence description and location of 25 chloroplast DNA primer pairs that did not amplify or did not have polymorphism.

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References

Adati, S., and I. Shiotani. 1962. The cytotaxonomy of the genus *Miscanthus* and its phylogenic status. Bull. Fac. Agric. Mie Univ. 25:1–14.

Al-Janabi, S.M., M. Mc Clelland, C. Petersen, and B.W.S. Sobral. 1994. Phylogenetic analysis of organellar DNA sequences in the *Andropogoneae: Saccharinae*. Theor. Appl. Genet. 88:933–944.

Calsa, T., Jr., D.M. Carraro, M.R. Benatti, A.C. Barbosa, J.P. Kitajima, and H. Carrer. 2004. Structural features and transcript-editing analysis of sugarcane (*Saccharum officinarum* L.) chloroplast genome. Curr. Genet. 46:366–373. doi:10.1007/s00294-004-0542-4

Chen, S.L., and S.A. Renvoize. 2006. *Miscanthus*. In: Z.Y. Wu, et al., editors, Flora of China, Poaceae. Science Press, Mo. Bot. Gard. Press, Beijing, St. Louis. p. 581–583.

Chiu, W.L., and B.B. Sears. 1985. Recombination between chloroplast DNAs does not occur in sexual crosses of *Oenothera*. Mol. Gen. Genet. 198:525–528. doi:10.1007/BF00332951

Clement, M., D. Posada, and K.A. Crandall. 2000. TCS: A computer program to estimate gene genealogies. Mol. Ecol. 9:1657–1659. doi:10.1046/j.1365-294x.2000.01020.x

Clifton-Brown, J.C., I. Lewandowski, B. Andersson, G. Basch, D.G. Christian, J.B. Kjeldsen, U. Jørgensen, J.V. Mortensen, A.B. Riche, K.-U. Schwarz, K. Tayebi, and F. Teixeira. 2001. Performance of 15 *Miscanthus* genotypes at five sites in Europe. Agron. J. 93:1013–1019. doi:10.2134/agronj2001.9351013x

Clifton-Brown, J.C., P.R.H. Robson, G.G. Allison, S.J. Lister, R. Sanderson, C. Morris, E. Hodgson, K. Farrar, S. Hawkins, E.F. Jensen, S.T. Jones, L. Huang, P.C. Roberts, S.J. Youell, B.R. Jones, A. Wright, J. Valentine, and I.S. Donnison. 2008. *Miscanthus*: Breeding our way to a better future. In: Aspects of Applied Biology, Biomass and Energy Crops III conference, Sand Hutton, UK. 10–12 Dec. 2008. p. 199–206.

De Cesare, M., T.R. Hodkinson, and S. Barth. 2010. Chloroplast DNA markers (cpSSRs, SNPs) for *Miscanthus*, *Saccharum* and related grasses (Panicoideae, Poaceae). Mol. Breed. 26:539–544. doi:10.1007/

s11032-010-9451-z
Dereeper, A., V. Guignon, G. Blanc, S. Audic, S. Buffet, F. Chevenet, J.-F. Dufayard, S. Guindon, V. Lefort, M. Lescot, J.-M. Claverie, and O. Gascuel. 2008. Phylogeny.fr: Robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. 36:465–469. doi:10.1093/nar/gkn180
Deuter, M. 2000. Breeding approaches to improvement of yield and quality in *Miscanthus* grown in Europe. In: European Miscanthus improvement. FAIR3 CT-96-1392. Final Report, September. Inst. of Crop Production and Grassland Res., Univ. of Hohenheim, Stuttgart. p. 28–52.
Dwiyanti, M.S., A. Rudolph, K. Swaminathan, A. Nishiwaki, Y. Shimono, S. Kuwabara, H. Matuura, M. Nadir, S. Moose, J.R. Stewart, and T. Yamada. 2013. Genetic analysis of putative triploid *Miscanthus* hybrids and tetraploid *M. sacchariflorus* collected from sympatric populations of Kushima, Japan. BioEnergy Res. 6:486–493. doi:10.1007/s12155-012-9274-3
Flannery, M.L., F.J.G. Mitchell, S. Coyne, T.A. Kavanagh, J.I. Burke, N. Salamin, P. Dowding, and T.R. Hodkinson. 2006. Plastid genome characterisation in *Brassica* and Brassicaceae using a new set of nine SSRs. Theor. Appl. Genet. 113:1221–1231. doi:10.1007/s00122-006-0377-0
Gielly, L., and P. Taberlet. 1994. The use of chloroplast DNA to resolve plant phylogenies—Noncoding versus *rbcl* sequences. Mol. Biol. Evol. 11:769–777.
Greef, J.M., and M. Deuter. 1993. Syntaxonomy of *Miscanthus*×*Giganthus* Greef-Et-Deu. Angew. Bot. 67:87–90.
Grivet, D., B. Heinze, G.G. Vendramin, and R.J. Petit. 2001. Genome walking with consensus primers: Application to the large single copy region of chloroplast DNA. Mol. Ecol. Notes 1:345–349. doi:10.1046/j.1471-8278.2001.00107.x
Hasegawa, M., H. Kishino, and T. Yano. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22:160–174. doi:10.1007/BF02101694
Heaton, E.A., F.G. Dohleman, and S.P. Long. 2008. Meeting US biofuel goals with less land: The potential of *Miscanthus*. Glob. Change Biol. 14:2000–2014. doi:10.1111/j.1365-2486.2008.01662.x
Hodkinson, T.R., M.W. Chase, and S.A. Renvoize. 2002a. Characterization of a genetic resource collection for *Miscanthus* (Saccharinae, Andropogoneae, Poaceae) using AFLP and ISSR PCR. Ann. Bot. (Lond.) 89:627–636. doi:10.1093/aob/mcf091
Hodkinson, T.R., M.W. Chase, M.D. Lledo, N. Salamin, and S.A. Renvoize. 2002b. Phylogenetics of *Miscanthus, Saccharum* and related genera (Saccharinae, Andropogoneae, Poaceae) based on DNA sequences from ITS nuclear ribosomal DNA and plastid *trnL* intron and *trnL-F* intergenic spacers. J. Plant Res. 115:381–392. doi:10.1007/s10265-002-0049-3
Hodkinson, T.R., M.W. Chase, C. Takahashi, I.J. Leitch, M.D. Bennett, and S.A. Renvoize. 2002c. The use of DNA sequencing (*ITS* and *trnL-F*), AFLP, and fluorescent in situ hybridization to study allopolyploid *Miscanthus* (Poaceae). Am. J. Bot. 89:279–286. doi:10.3732/ajb.89.2.279
Hodkinson, T.R., S.A. Renvoize, and MW Chase. 1997. Systematics of *Miscanthus*. Aspects Appl. Biol. 49:189–198.
Jiang, J.X., Z.H. Wang, B.R. Tang, L. Xiao, X. Ai, and Z.L. Yi. 2012. Development of novel chloroplast microsatellite markers for *Miscanthus* species (Poaceae). Am. J. Bot. 99:230–233. doi:10.3732/ajb.1100518
Jiang, J.X., M.D. Zhu, X. Ai, L. Xiao, G.T. Deng, and Z.L. Yi. 2013. Molecular evidence for a natural diploid hybrid between *Miscanthus sinensis* (Poaceae) and *M. sacchariflorus*. Plant Syst. Evol. 299:1367–1377. doi:10.1007/s00606-013-0801-2
Linde-Laursen, I. 1993. Cytogenetic analysis of *Miscanthus* 'Giganteus', an interspecific hybrid. Hereditas (Landskrona) 119:297–300. doi:10.1111/j.1601-5223.1993.00297.x
Ma, X.-E., E. Jensen, N. Alexandrov, M. Troukhan, L. Zhang, S. Thomas-Jones, K. Farrar, J. Clifton-Brown, I. Donnison, T. Swaller, and R. Flavell. 2012. High resolution genetic mapping by genome sequencing reveals genome duplication and tetraploid genetic structure of the diploid *Miscanthus sinensis*. PLoS ONE 7:1–11.
McGrath, S., T.R. Hodkinson, and S. Barth. 2007. Extremely high cytoplasmic diversity in natural and breeding populations of *Lolium* (Poaceae). Heredity 99:531–544. doi:10.1038/sj.hdy.6801030
Palmer, J.D., R.K. Jansen, H.J. Michaels, M.W. Chase, and J.R. Manhart. 1988. Chloroplast DNA variation and plant phylogeny. Ann. Mo. Bot. Gard. 75:1180–1206. doi:10.2307/2399279
Powell, W., M. Morgante, C. Andre, J.W. McNicol, G.C. Machray, J.J. Doyle, S.V. Tingey, and J.A. Rafalski. 1995a. Hypervariable microsatellites provide a general source of polymorphic DNA markers for the chloroplast genome. Curr. Biol. 5:1023–1029. doi:10.1016/S0960-9822(95)00206-5
Powell, W., M. Morgante, R. McDevitt, G.G. Vendramin, and J.A. Rafalski. 1995b. Polymorphic simple sequence repeat regions in chloroplast genomes: Applications to the population genetics of pines. Proc. Natl. Acad. Sci. USA 92:7759–7763. doi:10.1073/pnas.92.17.7759
Provan, J., W. Powell, and P.M. Hollingsworth. 2001. Chloroplast microsatellites: New tools for studies in plant ecology and evolution. Trends Ecol. Evol. 16:142–147. doi:10.1016/S0169-5347(00)02097-8
Quinn, L.D., T.M. Culley, and J.R. Stewart. 2012. Genetic comparison of introduced and native population of *Miscanthus sinensis* (Poaceae), a potential bioenergy crop. Grassl. Sci. 58:101–111. doi:10.1111/j.1744-697X.2012.00248.x
Su, Y., T. Wang, B. Zheng, Y. Jiang, G. Chen, and H. Gu. 2004. Population genetic structure and phylogeographical pattern of a relict tree fern, *Alsophila spinulosa* (Cyatheaceae), inferred from cpDNA *atp*B-*rbc*L intergenic spacers. Theor. Appl. Genet. 109:1459–1467. doi:10.1007/s00122-004-1761-2
Sun, Q.A., Q. Lin, Z.L. Yi, Z.R. Yang, and F.S. Zhou. 2010. A taxonomic revision of *Miscanthus s.l.* (Poaceae) from China. Bot. J. Linn. Soc. 164:178–220. doi:10.1111/j.1095-8339.2010.01082.x
Templeton, A.R., K.A. Crandall, and C.F. Sing. 1992. A cladistic-analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. 3. Cladogram estimation. Genetics 132:619–633.
Zub, H.W., S. Arnoult, and M. Brancourt-Hulmel. 2011. Key traits for biomass production identified in different *Miscanthus* species at two harvest dates. Biomass Bioenergy 35:637–651. doi:10.1016/j.biombioe.2010.10.020
Zub, H.W., and M. Brancourt-Hulmel. 2010. Agronomic and physiological performances of different species of *Miscanthus*, a major energy crop. A review. Agron. Sustain. Dev. 30:201–214. doi:10.1051/agro/2009034
Zub, H.W., C. Rambaud, L. Béthencourt, and M. Brancourt-Hulmel. 2012. Late emergence and rapid growth maximize the plant development of *Miscanthus* clones. BioEnergy Res. 5:841–854. doi:10.1007/s12155-012-9194-2