Plant Syst Evol (2013) 299:1367–1377
DOI 10.1007/s00606-013-0801-2

ORIGINAL ARTICLE

Molecular evidence for a natural diploid hybrid between *Miscanthus sinensis* (Poaceae) and *M. sacchariflorus*

Jianxiong Jiang · Mingdong Zhu · Xin Ai · Liang Xiao · Guote Deng · Zili Yi

Received: 12 June 2012 / Accepted: 20 March 2013 / Published online: 7 April 2013

Abstract The hybrid origin of *Miscanthus purpurascens* has previously been proposed, primarily because of its intermediate morphology. In this study, phylogenies based on the DNA sequences from the internal transcribed spacer region of nuclear ribosomal DNA (nrDNA ITS), on the DNA sequences of the *trn*L intron and *trn*L-F intergenic spacer of chloroplast DNA, and on amplified fragment length polymorphism (AFLP) fingerprinting confirm that *M. purpurascens* originated through homoploid hybridization between *M. sinensis* and *M. sacchariflorus*. Two different types of ITS sequences were identified from almost all plants of *M. purpurascens*. One type was found to be closely related to *M. sinensis* and the other to *M. sacchariflorus*. *Miscanthus purpurascens* was found to possess many *M. sinensis*- and *M. sacchariflorus*-specific AFLP bands but no band specific to itself. Clustering with the Unweighted Pair Group Method with Arithmetic Mean and principal coordinate analysis based on the AFLP data also demonstrated that *M. purpurascens* is an approximate intermediate of the two species. In addition, *M. purpurascens* has the plastid genome of *M. sinensis* or *M. sacchariflorus*, suggesting that either species could be its maternal parent. All specimens of *M. purpurascens* and its coexisting parental species are identified as diploids ($2n = 2x = 38$). Possible mechanisms of natural hybridization, hybrid status, chloroplast DNA recombination, and evolutionary implications of this hybridization are also discussed.

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Keywords AFLP · Homoploid hybridization · ITS · *Miscanthus purpurascens* · *rpl*16 · *trn*L-F

Introduction

Spontaneous interspecific hybridization is widespread in plants due to incomplete reproductive isolation between closely related species. It has long been recognized as an evolutionary force capable of creating genetic novelties that promote adaptive evolution and speciation (Arnold 1997, 2004; Rieseberg 1997; Seehausen 2004). It is estimated that approximately 50 % of angiosperm species are of hybrid origin (Arnold 1997). Usually, hybrid speciation is accomplished through either of two different mechanisms: allopolyploid hybrid speciation (in which the hybrid species has a higher ploidy level than either parent) or homoploid hybrid speciation (in which the hybrid species has the same number of chromosomes as either parent). Homoploid hybrid speciation involves hybridization between plant taxa at the same ploidy level and is considered rarer than allopolyploid hybrid speciation. It is achieved through ecological and spatial reproductive isolations or chromosomal rearrangement within the hybrid (reviewed in Abbott et al. 2010). Recently, more and more examples of natural homoploid hybrid species in plants have been reported (Ferguson and Sang 2001; Gammon et al. 2007; Gross et al. 2003; Nagamitsu et al. 2006; Pan et al. 2007). These provide opportunities for investigation of the different ways in which they originate.

Miscanthus Andersson (Poaceae) is a perennial rhizomatous grass with a C4 photosynthetic pathway. Most *Miscanthus* species are native to East Asia. In recent years, *Miscanthus* has attracted considerable attention for its potential as a bioenergy crop (Heaton et al. 2004; Zub and

Brancourt-Hulmel 2010). *Miscanthus x giganteus*, which was introduced to Europe from Japan as an ornamental grass in 1935, has been extensively cultivated and studied in Europe and North America for biomass feedstock production because of its high biomass yield and relatively low energy and financial input requirements (Bullard et al. 1997; Collura et al. 2006; Heaton et al. 2008; Lewandowski et al. 2000). Based on its morphological and cytological characteristics, it has been hypothesized that *M. x giganteus* may be an allotriploid hybrid ($2n = 3x = 57$) that originated from natural hybridization between *M. sinensis* and *M. sacchariflorus* (Greef and Deuter 1993; Hodkinson and Renvoize 2001; Linde-Laursen 1993). Evaluation of the interrelationship between *M. x giganteus* and other *Miscanthus* taxa using amplified fragment length polymorphism (AFLP) fingerprinting supported this hypothesis (Hodkinson et al. 2002a). Analyses of the DNA sequences from the internal transcribed spacer region of nuclear ribosomal DNA (nrDNA ITS) and the *trn*L intron and *trn*L-F intergenic spacer of chloroplast DNA (cpDNA) confirmed the hybrid origin of *M. x giganteus*, suggesting that *M. sacchariflorus* was the maternal species of *M. x giganteus* (Hodkinson et al. 2002b). However, fluorescent in situ hybridization (FISH) and genomic in situ hybridization (GISH) failed to differentiate the different parental genomes present in *M. x giganteus*, suggesting pronounced similarity in the repetitive DNA sequences (Hodkinson et al. 2002b), and it remains unclear which parental species provides two genomes to *M. x giganteus*. Until recently, no other natural hybrid in *Miscanthus* has been recognized or reported.

Here, we report molecular evidence supporting the origin of a diploid hybrid through homoploid hybridization between *M. sinensis* and *M. sacchariflorus*. *Miscanthus purpurascens* was described as a separate species by Liu (1997) in Flora of China, and it is mainly characterized by the purple callus hairs at the base of spikelet, different from the white or yellow callus hairs in other *Miscanthus* taxa. But Chen and Renvoize (2006) believed that difference in callus hair color is not a sufficient evidence for distinguishing *M. purpurascens* from *M. sinensis*. Recently, Sun et al. (2010) further considered that callus hair color is not an ideal taxonomic character, because it can become white from purple gradually during *Miscanthus* panicle development. Thus, the taxonomic status of *M. purpurascens* is in doubt. During our previous field investigations of wild *Miscanthus* germplasms in 2006–2009, we found *M. purpurascens* to be mainly spread in Liaoning and southern Jilin areas and to be sympatric with *M. sinensis* and *M. sacchariflorus*. These three taxa usually coexist in small, mixed populations at some locations. This is because the ability of *Miscanthus* to reproduce sexually is weak. *Miscanthus sinensis* and *M. sacchariflorus* are distinct in their morphology (Chen and Renvoize 2006). However, *M. purpurascens* exhibits several intermediate morphological characters suggestive of both *M. sinensis* and *M. sacchariflorus*. This allows us to speculate that *M. purpurascens* may be of hybrid origin. In this study, molecular data of nrDNA ITS sequences and AFLP markers from the above-mentioned three *Miscanthus* taxa were analyzed to confirm the hybrid origin of *M. purpurascens*. The maternally inherited chloroplast *trn*L-F intergenic spacer and *rpl*16 intron region were also sequenced to identify the putative maternal parent species for *M. purpurascens*. In addition, the conventional root tip squashing technique was used to determine the chromosome number and ploidy level of the *Miscanthus* samples. The purpose of this study was to determine the hybrid nature of *M. purpurascens* and assess its hybrid status.

Materials and methods

Plant materials

Twenty-nine specimens including nine *M. purpurascens*, ten *M. sinensis*, and ten *M. sacchariflorus* were sampled from one pure *M. purpurascens* population, three pure *M. sinensis* populations, three pure *M. sacchariflorus* populations, and five mixed populations located in Liaoning and Jilin Provinces in China (Fig. 1; Table 1). *Miscanthus sinensis* has short vertically oriented rhizomes, clustered culms without nodal buds, and a long lemma awn of 10–17 mm. *Miscanthus sacchariflorus* is awnless but has long horizontally oriented rhizomes and scattered culms with nodal buds that usually develop into branches. *Miscanthus purpurascens* exhibits a short lemma awn of 1–6 mm and has medium-sized rhizomes and clustered culms with nodal buds that rarely produce branches. Rhizomes from individuals were collected from sampling locations and grown in the *Miscanthus* Germplasm Nursery of Hunan Agricultural University. Voucher specimens were deposited in the Herbarium of *Miscanthus* Research Institute, Hunan Agricultural University.

DNA sequencing

Total genomic DNA was extracted from young leaves using a CTAB procedure (Doyle and Doyle 1987). The ITS_1-5.8S-ITS_2 region of nuclear ribosomal DNA was amplified from all specimens using the universal primers "ITS4" (5′-TCC TCC GCT TAT TGA TAT GC-3′) and "ITS5" (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) (White et al. 1990). The chloroplast *trn*L-F intergenic spacer region was amplified with the primers "*c*" (5′-CGA AAT CGG TAG ACG CTA CG-3′) and "*f*" (5′-ATT

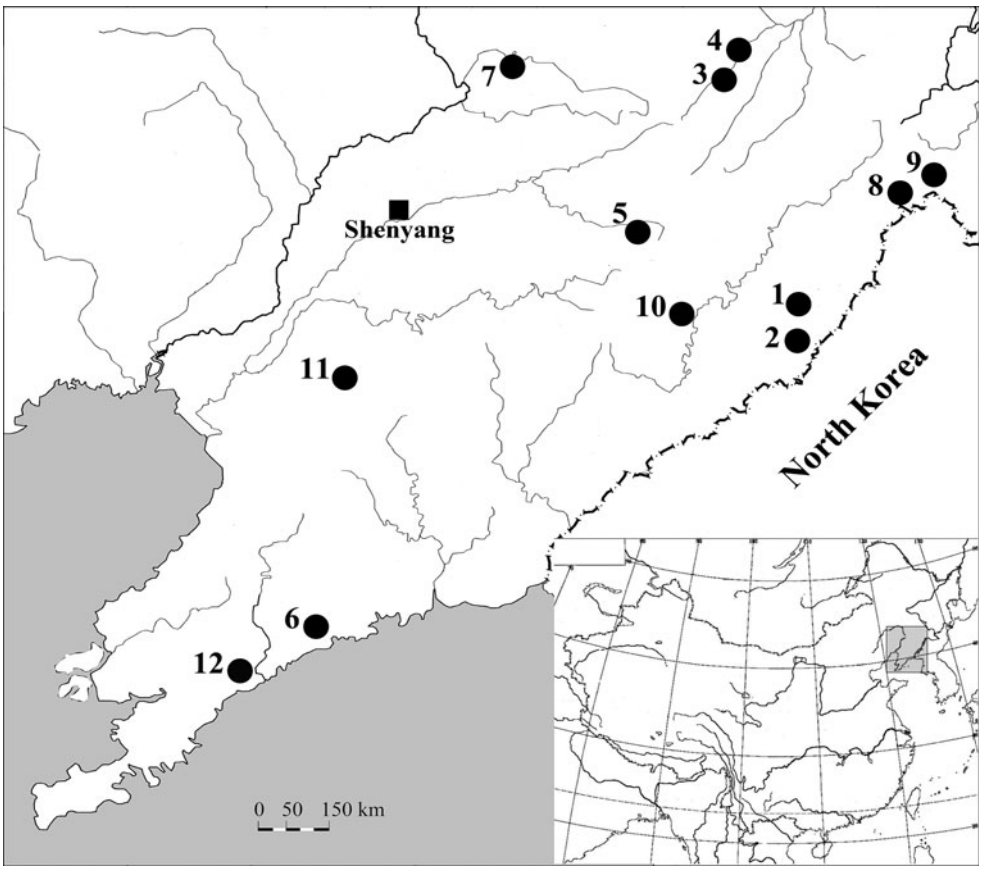

Fig. 1 Map of the locations from which *Miscanthus purpurascens* and its putative parental species were collected for this study. Numbers correspond to location numbers of the populations given in Table 1

TGA ACT GGT GAC ACG AG-3′) described by Taberlet et al. (1991). The chloroplast *rpl*16 intron region was amplified as described by Jordan et al. (1996) with the primers "*rpl*16-F71" (5′-GCT ATG CTT AGT GTG TGA CTC GTT G-3′) and "*rpl*16-R1516" (5′-CCC TTC ATT CTT CCT CTA TGT TG-3′). PCR products were separated through 1 % agarose gel electrophoresis and then purified using the E.Z.N.A.™ Gel Extraction Kit (Omega Bio-Tek, Doraville, GA, USA). Direct sequencing was conducted from both strands to produce consensus sequences on an ABI 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) by Shanghai Sunny Biotechnology, Inc. Considering the possible heterogeneity within ITS sequences, a T-A cloning step was also performed for ITS PCR products using the InsTAclone™ Cloning Kit (Fermentas, Burlington, Ontario, Canada) and 10–20 positive bacterial colonies for each individual were sequenced. Corresponding DNA fragments in *M. paniculatus*, *M. nepalensis*, and *M. nudipes* were also sequenced and used as outgroups in the phylogenetic analyses. All DNA sequences for this study have been deposited in GenBank (Accession numbers JN544243–JN544347).

DNA sequences were aligned using ClustalX 1.83 (Thompson et al. 1997) and BioEdit 7.0.8 (Ibis Biosciences, Carlsbad, CA, USA). Gap information in the sequences was coded and added to the data matrix using the method described by Simmons and Ochoterena (2000) using the Gapcoder software (Yong and Healy 2003). Chloroplast *trn*L-F and *rpl*16 sequences were aligned individually and then concatenated into a single, contiguous sequence for each specimen. Molecular phylogenies between taxa were reconstructed using the neighbor-joining (NJ) and Bayesian inference (BI) methods. NJ analysis was conducted using MEGA 4 (Tamura et al. 2007) with a Kimura 2-parameter distance model (Kimura 1980) and bootstrap analysis used 1,000 replicates. BI analysis was performed in MrBayes 3.1 (Huelsenbeck and Ronquist 2001). jModelTest 0.1.1 (Posada 2008) was used to estimate the best-fit model of nucleotide substitution including rate heterogeneity under the Akaike Information Criterion (AIC; Akaike 1974). The best-fit model for ITS, *trn*L-F and *rpl*16 was TIM3+I, F81 and F81+G, respectively. Chloroplast concatenated dataset was partitioned by locus, with the previously inferred best-fit model for each locus. The Markov Chain Monte Carlo (MCMC) analysis consisted of two independent runs starting from random trees with four chains that were run for 2,000,000 generations and sampled every 1,000 generations. Topological convergence between runs was assessed using the online program AWTY

Table 1 Sampling information corresponding to sample labels in Fig. 1

Population	Source	Accession no.	Taxon	Voucher
1	Wunufeng, Ji'an, Jilin, China	A	*M. sinensis*	HN2381
		B	*M. sinensis*	HN2382
2	Maxian, Ji'an, Jilin, China	A	*M. purpurascens*	HN2384
		B	*M. sacchariflorus*	HN2385
3	Sancheng, Meihekou, Jilin, China	A	*M. sacchariflorus*	HN2400
		B	*M. sinensis*	HN2401
4	Jinhua, Meihekou, Jilin, China	A	*M. purpurascens*	HN2403
5	Hongshen, Xinbing, Liaoning, China	A	*M. sacchariflorus*	HN2391
		B	*M. sinensis*	HN2392
		C	*M. purpurascens*	HN2393
		D	*M. sinensis*	HN2394
		E	*M. sacchariflorus*	HN2395
6	Xuling, Zhuanghe, Liaoning, China	A	*M. sinensis*	HN2436
		B	*M. purpurascens*	HN2437
		C	*M. purpurascens*	HN2438
		D	*M. sacchariflorus*	HN2439
7	Qinghe, Tieling, Liaoning, China	A	*M. sacchariflorus*	HN2453
		B	*M. purpurascens*	HN2454
		C	*M. sinensis*	HN2455
		D	*M. purpurascens*	HN2456
		E	*M. sinensis*	HN2457
		F	*M. purpurascens*	HN2458
		G	*M. sacchariflorus*	HN2459
		H	*M. purpurascens*	HN2460
8	Heisong, Linjiang, Jilin, China	A	*M. sacchariflorus*	HN2361
9	Dahu, Linjiang, Jilin, China	A	*M. sinensis*	HN2366
10	Yahe, Huanren, Liaoning, China	A	*M. sacchariflorus*	HN2389
11	Jicuimen, Anshan, Liaoning, China	A	*M. sinensis*	HN2416
12	Chengzitan, Pulandian, Liaoning, China	A	*M. sacchariflorus*	HN2435
Outgroups	Sujia, Zhaotong, Yunnan, China		*M. paniculatus*	HN3181
	Madeng, Jianchuan, Yunnan, China		*M. nepalensis*	HN3176
	Xishan, Kunming, Yunnan, China		*M. nudipes*	HN3178

(Nylander et al. 2008). The samples generated before reaching stationarity in each run were discarded as burn-in and the remaining samples were used to calculate posterior probabilities.

AFLP analysis

AFLP analysis was performed using a fluorescent AFLP Kit (Dingguochangsheng Biotechnology, Inc., Beijing, China) in accordance with the manufacturer's instruction. Genomic DNA (200 ng) was double-digested with *Hind*III and *Mse*I restriction enzymes and then ligated to *Hind*III and *Mse*I adapters. The digestion-ligation mixture was preamplified by PCR using a *Hind*III adapter primer (5′-AGA CTG CGT ACC AGC TTA-3′) and an *Mse*I adapter primer (5′-GAT GAG TCC TGA GTA AC-3′). A 1:10 diluted aliquot of the pre-amplified mix was used as the template for selective amplification. Combinations of four *Hind*III primers (H-AAC, -ACA, -ACG, and -AGC) and two FAM fluorescence-labeled *Mse*I primers (M-CAA and -CAC) were employed in selective amplification. The selective PCR products were separated through 4 % denaturing polyacrylamide gel electrophoresis in an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). AFLP analysis was repeated twice, and only reproducible DNA fragments ranging from 50 to 500 base pairs were scored with GeneScan 3.1 software (Applied Biosystems, Foster City, CA, USA) and Binthere 1.0 software (Garnhart 2001). AFLP data were analyzed using NTSYSpc 2.1e software

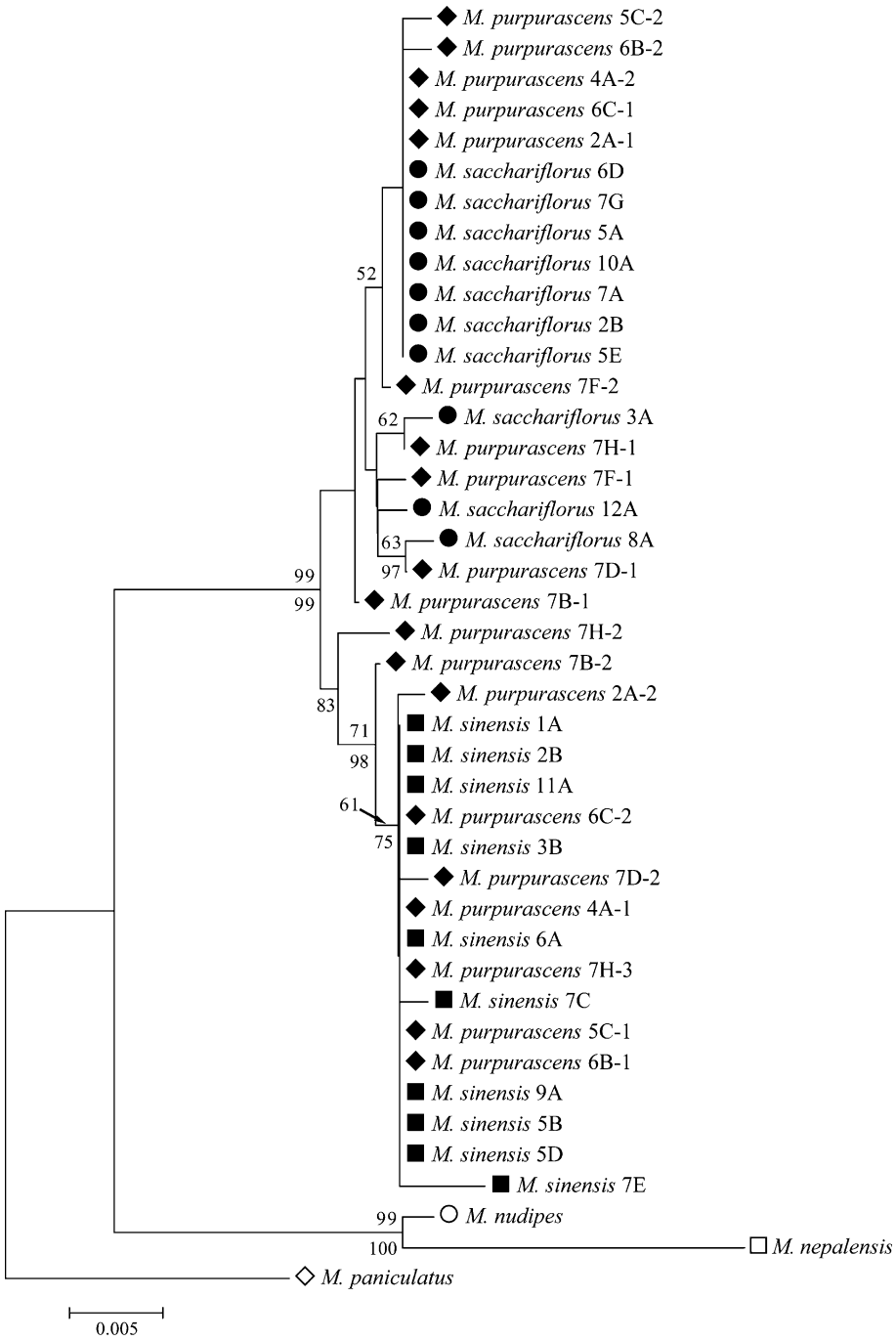

Fig. 2 The phylogeny of *Miscanthus purpurascens*, *M. sinensis*, and *M. sacchariflorus* obtained from DNA sequences of the internal transcribed spacer region of nuclear ribosomal DNA. *Numbers above* and *below* branches represent the relative bootstrap values >50 % and the posterior probability multiplied by 100 and >70, respectively. Accession numbers follow their corresponding taxon names. Clone numbers follow *hyphens*

(Exeter Software, Setauket, NY, USA) to determine the Dice similarity coefficient matrix. Using this matrix, a dendrogram was constructed using clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). A principal coordinate analysis (PCoA) was performed with the MVSP 3.1 software (Kovach Computing Services, Anglesey, Wales, UK).

Chromosome number and ploidy level

Root tips 0.5–1.0 cm long were pretreated at 10 °C for 2 h with a mixture of 0.2 % colchicine and 0.002 mol L^{-1} 8-hydroxyquinoline. They were then fixed in 3:1 alcohol-acetic acid solution at 4 °C for 20 h. The fixed samples were hydrolyzed with 1 mol L^{-1} hydrochloric acid at 60 °C for

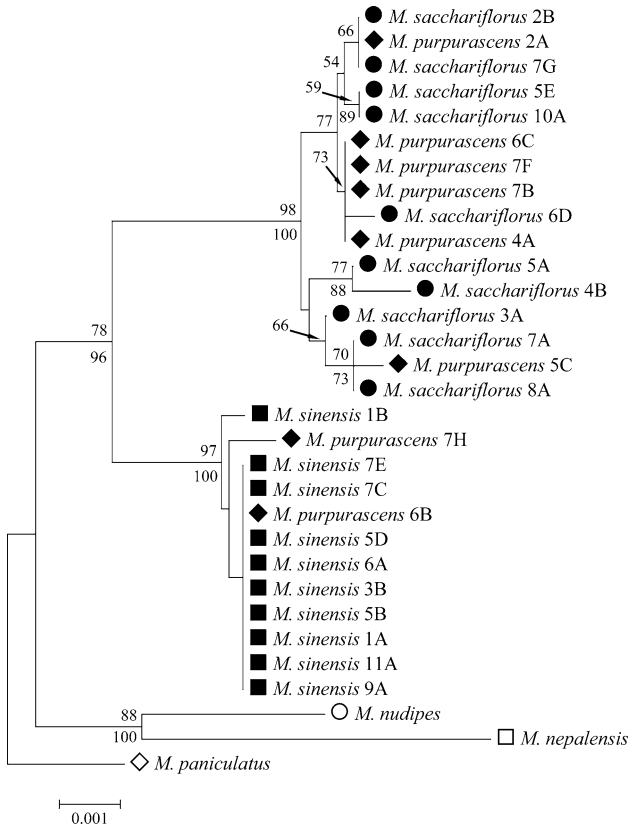

Fig. 3 The phylogeny of *Miscanthus purpurascens*, *M. sinensis*, and *M. sacchariflorus* obtained from combined chloroplast *trnL-F* intron spacer and *rpl16* intron sequences. *Numbers above* and *below* branches represent relative bootstrap values >50 % and the posterior probability multiplied by 100 and >70, respectively

Table 2 Number of AFLP bands specific to each *Miscanthus* taxon

P1–P2 shared	140
P1–P2–H shared	140
P1 specific	65
P1–H shared	28–36 (32.1)
P2 specific	68
P2–H shared	22–36 (28.7)
H specific	0

P1, *M. sinensis*; P2, *M. sacchariflorus*; H, *M. purpurascens*

15 min and 2 % cellulase at 28 °C for 1 h and then squashed in 45 % acetic acid and stained with 0.5 % modified carbol fuchsin. Over 30 well-spread cells were examined to determine the number of chromosomes for each sample.

Results

Phylogenetic analyses based on DNA sequence data

The topology of phylogenetic trees from the neighbor-joining and Bayesian analyses of ITS or combined chloroplast DNA (*trn*L-F + *rpl*16) data set is consistent. Only the NJ trees are shown here. NJ bootstrap support >50 % and BI posterior probabilities >0.7 are indicated by numbers above and below the branches, respectively.

Of the 661 characters in the aligned ITS matrix, 34 singleton variable sites, 16 parsimony informative sites, and 5 indel sites were identified. In the ITS phylogenies, the cloned sequences from samples of *M. sinensis* and *M. sacchariflorus* clustered into two distinct species-clades with a strong branch support (Fig. 2). However, two divergent types of ITS sequences were identified from all *M. purpurascens* samples except one individual (7F). One sequence was found to be closely related to the *M. sinensis* type and another to the *M. sacchariflorus* type. The ITS clones of *M. purpurascens* were divided between the *M. sinensis* clade and the *M. sacchariflorus* clade in the phylogenetic tree but they did not form a monophyletic subclade (Fig. 2). In the clones from 7F, only *M. sacchariflorus* ITS sequences were detected.

The aligned combined chloroplast sequence (*trn*L-F + *rpl*16) had a length of 2,083 characters, with 18 singleton variable sites, 11 parsimony informative sites, and 148 indel sites. In the chloroplast DNA phylogeny, the accessions of *M. sinensis* and *M. sacchariflorus* were also grouped into two well-supported species-clades, as did the ITS phylogeny (Fig. 3). Two specimens of *M. purpurascens* from populations 6 (6B) and 7 (7H) formed a monophyletic group with *M. sinensis*, and the other six specimens of *M. purpurascens* (2A, 4A, 5C, 6C, 7B, and 7F) were clustered together with *M. sacchariflorus* (Fig. 3).

One specimen of *M. purpurascens* (7D) was excluded from the phylogenetic analysis of chloroplast DNA because its chloroplast genome was a recombinant of *M. sinensis* and *M. sacchariflorus* (data not shown). This chloroplast DNA recombinant possessed a single-nucleotide polymorphism (SNP) unique to *M. sinensis* and one SNP and one indel unique to *M. sacchariflorus* in its *trn*L-F sequence. It also had one SNP and one indel unique to *M. sinensis* and three SNP unique to *M. sacchariflorus* in its *rpl*16 sequence. Chloroplast DNA homologous recombination events have been reported in interspecific hybridization of *Chlamydomonas* (Lemieux et al. 1981, 1984) and in somatic hybridization of *Nicotiana* (Medgyesy et al. 1985).

Phylogenetic analyses based on AFLP data

AFLP analysis was conducted to assess the hybrid status of *M. purpurascens*. A total of 657 bands were identified using eight selective primer combinations that discriminated 29 specimens of *M. purpurascens*, *M. sinensis*, and *M. sacchariflorus*. Among those, 140 bands were found to be common to all specimens analyzed. There were 65 monomorphic, species-specific bands present in all

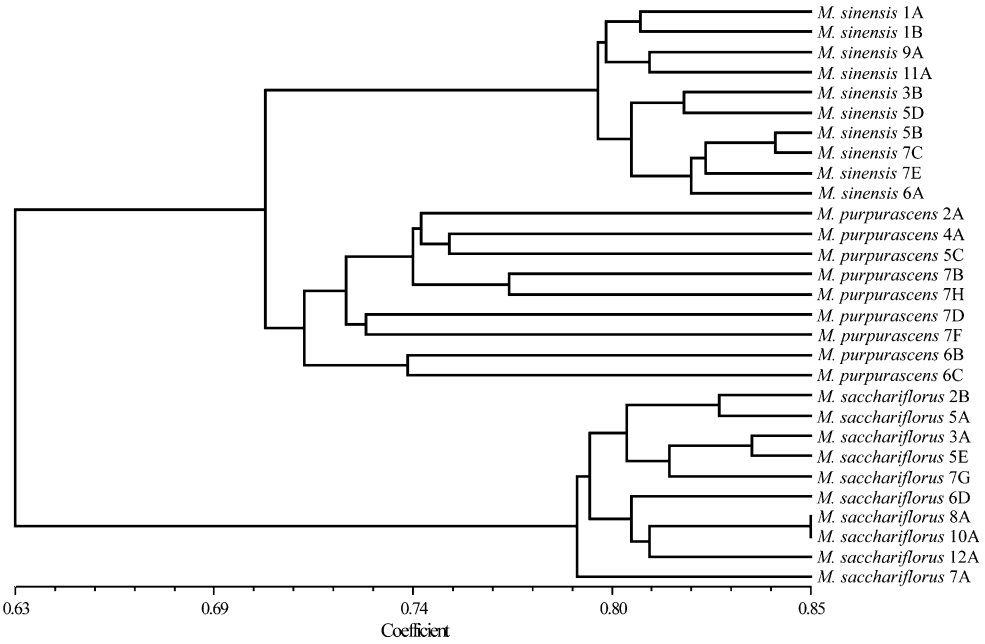

Fig. 4 Dendrogram showing genetic relationships among *Miscanthus purpurascens*, *M. sinensis*, and *M. sacchariflorus* based on amplified fragment length polymorphism (AFLP) markers. The dendrogram was constructed using clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA)

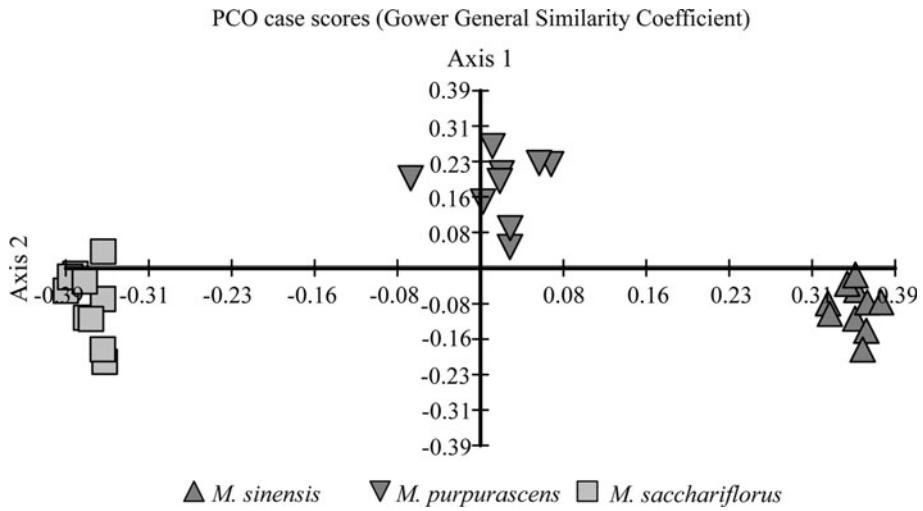

Fig. 5 Principal coordinates analysis (PCoA) of amplified fragment length polymorphism (AFLP) data for individuals of *Miscanthus purpurascens*, *M. sinensis* and *M. sacchariflorus*. *Axis 1* and *axis 2* explain 27.02 and 5.46 % of the total variation, respectively

M. sinensis specimens and 68 monomorphic species-specific bands in all *M. sacchariflorus* specimens, but no bands unique to *M. purpurascens* were detected. *Miscanthus purpurascens* specimens possessed 28–36 (mean = 32.1) *M. sinensis*-specific bands and 22–36 (mean = 28.7) *M. sacchariflorus*-specific bands (Table 2). Two *M. sinensis*-specific bands and four *M. sacchariflorus*-specific bands were absent from all *M. purpurascens* specimens.

The average genetic similarity index between *M. sinensis* and *M. purpurascens* was 0.6333, but the average genetic similarity index between *M. sacchariflorus* and *M. purpurascens* was 0.6157. The UPGMA tree based on AFLP data comprised three distinct primary clusters corresponding to the three taxa recognized by morphology. The cluster representing *M. purpurascens* was approximately equidistant between the *M. sinensis* cluster and the *M. sacchariflorus* cluster (Fig. 4). The intermediacy of *M. purpurascens* was also demonstrated by PCoA, in which the three taxa were completely discriminated along the first axis (Fig. 5).

Chromosome number and ploidy level analysis

Karyotyping of root tip cells revealed that all specimens of *M. purpurascens*, *M. sinensis*, and *M. sacchariflorus* were diploids with a chromosome number of $2n = 2x = 38$ (Fig. 6).

Discussion

The hybrid origin of *M. purpurascens* from hybridization between *M. sinensis* and *M. sacchariflorus* has been confirmed in this study. First, both ITS sequence types from

parental species are present in the same individuals of *M. purpurascens* plants, suggesting that the hybrids originated so recently that concerted evolution has not yet been able to homogenize their nuclear ribosomal loci. The absence of *M. sinensis* ITS sequences from *M. purpurascens* 7F may have been caused by an insufficient number of sequenced clones, but an alternative explanation could be biased gene conversion (Hillis et al. 1991). In a hybrid scallop system (*Chlamys farreri* × *Argopecten irradians*), rapid, concerted gene evolution of the ITS regions via maternally biased gene conversion was observed, causing the absence of paternal ITS sequences from the hybrids (Wang et al. 2010). In other cases, the ribosomal repeats were homogenized to one parental type or a recombinant intermediate following hybridization (Franzke and Mummenhoff 1999; Hughes and Petersen 2001; Wendel et al. 1995). Second, *M. purpurascens* was found to have the chloroplast genome of either *M. sinensis* or *M. sacchariflorus*, indicating reciprocal hybridization between the two species. Third, *M. purpurascens* additively inherited 28–36 *M. sinensis*-specific and 22–36 *M. sacchariflorus*-specific AFLP bands. Both the UPGMA and PCoA analyses indicated that all *M. purpurascens* specimens showed values approximately halfway between those of *M. sinensis* and those of *M. sacchariflorus*.

Miscanthus sinensis and *M. sacchariflorus* are sympatric in northern and northeastern China, but *M. sacchariflorus* populations are denser and this species is distributed farther north than *M. sinensis* because its long, robust rhizomes help it to survive through freezing, drought, and alkaline conditions. Northern China is a heavily populated plains region and most of its land has been reclaimed for agriculture use. Agricultural encroachment has almost driven *M. sinensis* to extinction, but *M. sacchariflorus* can survive and grow along the edges of fields. In contrast, both species can survive and coexist in hilly regions of northeastern China (especially Southeastern Liaoning and southern Jilin), which provides an opportunity for natural hybridization. Another factor contributing to natural hybridization is their overlapping flowering periods. *Miscanthus sacchariflorus* blooms from late August to September and *M. sinensis* blooms slightly later, and there is about 1 month of overlap between the two species. This suggests that hybridization can occur. Southeastern Liaoning and southern Jilin areas belong to a wider Changbaishan Mountain system, with a very rich forest. *Miscanthus* species are pioneer plants that are able to colonize the vacant or newly reclaimed lands, and in the process of ecological succession they are gradually pushed away by shade trees, which overshadow them. In addition, *Miscanthus* species have low sexual reproductive capacity and low seed fitness. These characteristics limited the dispersal and then the population size of *M. purpurascens* in most habitats. An exception is the population 7 from Qinghe, Liaoning, which is situated on a gradual slope at the edge of a westward trend of the Changbaishan

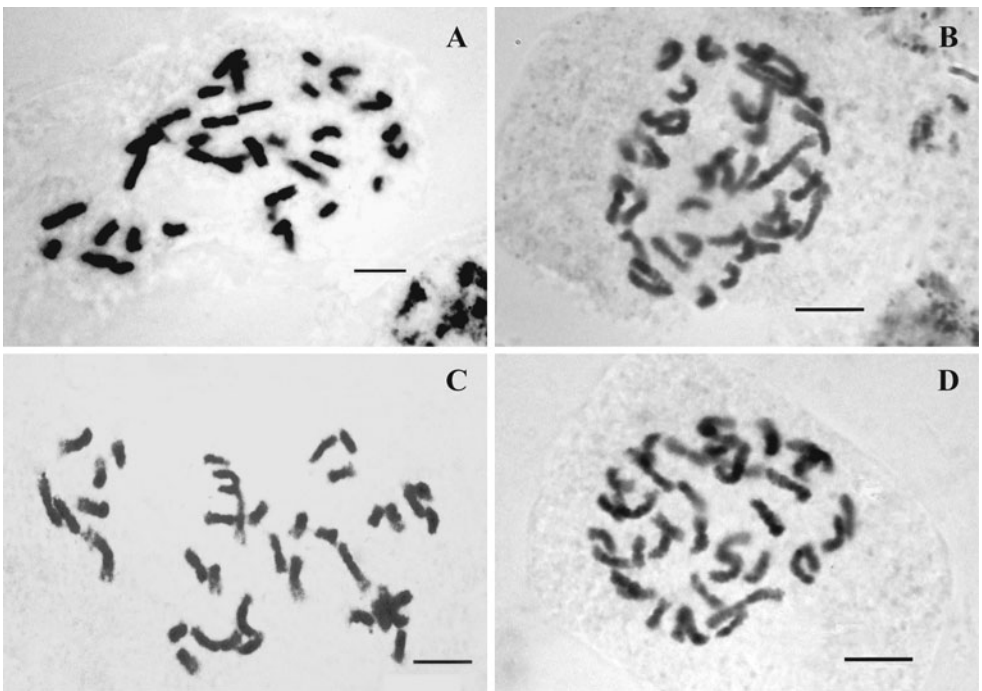

Fig. 6 The chromosome number of *Miscanthus purpurascens* and its coexisting parental species (*bar* 10 μm). **a** *M. sinensis* 1A ($2n = 2x = 38$); **b** *M. sacchariflorus* 5A ($2n = 2x = 38$); **c–d** *Miscanthus purpurascens* 6B and 7D ($2n = 2x = 38$)

Mountain system toward the Northeast Plain. The slope is an uncultivated south-facing land with dwarf shrubs, which facilitates pollination and hybridization of *Miscanthus*. Therefore, this population comprises dozens of *M. purpurascens* plants. Maybe more populations like this can be found in plain regions, but these populations are still fragile because of possible human disturbance.

All specimens of *M. purpurascens* and coexisting parental species presented in this study were detected as diploids ($2n = 2x = 38$), suggesting that *M. purpurascens* originated through hybridization between the diploid species *M. sinensis* and *M. sacchariflorus*. *M. x giganteus*, which was previously the only known *Miscanthus* hybrid, is an allotriploid hybrid ($2n = 3x = 57$) between *M. sinensis* and *M. sacchariflorus* (Greef and Deuter 1993; Linde-Laursen 1993; Hodkinson and Renvoize 2001). The present study revealed a novel form of hybrid speciation in *Miscanthus*, one other than allopolyploid hybrid speciation. So far, the genome composition of *M. x giganteus* is still unknown because neither FISH nor GISH can differentiate the parental genomes present in *M. x giganteus* because of the pronounced similarity of repetitive DNA sequences between the two parental species (Hodkinson et al. 2002b; Takahashi and Shibata 2002). Compared to *M. purpurascens*, *M. x giganteus* has a morphology bias toward *M. sacchariflorus*, suggesting that the allotriploid should have two sets of *M. sacchariflorus* genomes, that it is a hybrid between 4*x* *M. sacchariflorus* and 2*x* *M. sinensis*. This conclusion is also supported by the flow cytometry analysis conducted by Rayburn et al. (2009). That analysis indicated that the hypothetical hybrid between 4*x* *M. sinensis* and 2*x* *M. sacchariflorus* was estimated to have a nuclear DNA content of 7.75 pg, and the hybrid between 4*x* *M. sacchariflorus* and 2*x* *M. sinensis* would have a DNA content of 7.2 pg, which corresponds more closely to the observed nuclear DNA content of *M. x giganteus* (7.0 pg).

AFLP analysis proved to be a reliable method for assessing hybrid status (Kameyama et al. 2005; Teo et al. 2002; Zhou et al. 2005). Because AFLP is a dominant marker, a real hybrid will additively express the bands specific to either of its parental species. For example, an F1 generation will always be heterogeneous for each set of parental species-specific AFLP bands, and 100 % of these would be expressed. With one backcross, the proportion of parental species-specific bands observed in BC1 generation would be 100 % for one parental species and 50 % for the other. Similarly, the proportion of parent-specific AFLP bands for either parental species would be 75 % in F2 generation, 50 % in F3 generation, and so on. The AFLP analysis in this study showed that only 43.1–55.4 % of *M. sinensis*-specific bands and 32.4–52.9 % of *M. sacchariflorus*-specific bands expressed in each genotype of *M. purpurascens* and, therefore, *M. purpurascens* would most likely be the hybrids of F3 generation. However, *Miscanthus* is self-incompatible, resulting in highly heterozygous alleles. Because we cannot distinguish between homozygous and heterozygous status of the dominant AFLP markers, the absence of some parental species-specific bands from these hybrids may be caused by parental heterozygosity and consequent homozygous recessive status of hybrids (Jug et al. 2004). For this reason, the possibility that an individual *M. purpurascens* would be an F1 or F2 hybrid cannot be excluded. The most likely hypothesis is that *M. purpurascens* is a sterile F1 hybrid. This is because a hybrid swarm (a continuum of morphological variation between two parental species) created by intercrossing among the hybrids was not observed in the population. In this case, the diploid hybrid *M. purpurascens* cannot be established as a novel species from the viewpoint of sexual reproduction. At present, it is impossible to test the fertility of *M. purpurascens* because the *Miscanthus* plants collected from northeastern China cannot flower normally when grown in Hunan. However, some pre- and post-zygotic barriers may be involved in the reproductive isolation between the hybrids and their parental species. For example, the seed-set rate of *M. purpurascens*, which was tested using several panicles collected from original sampling locations, ranged only from 1.7 to 3.2 %. Almost none of the seeds were able to germinate, most of them being empty or shriveled (data not shown).

In general, F1 hybrids of closely related species tend to exceed their parents in vegetative growth vigor, also called robustness. The transgressive changes in phenotypic traits and gene expression are of great importance in the adaptation of homoploid hybrid species to habitats that are ecologically and spatially different from those of their parents, allowing the hybrids to compete with or even replace their parental species (reviewed by Les and Philbrick 1993). The reproduction of *Miscanthus* mainly depends on clonal propagation via branched rhizomes, and *M. purpurascens* has a more robust rhizome system than *M. sinensis*, implying that it is well adapted to the increasingly serious drought and alkaline conditions in northeastern China, as reflected in the fact that *M. purpurascens* outnumbers *M. sinensis* in mixed populations. However, a more detailed study is required to determine the relationship between the hybrid vigor of *M. purpurascens* and habitat adaptation.

The inheritance of chloroplast DNA in *Miscanthus* was assumed to be maternal (Hodkinson et al. 2002b). However, the chloroplast genome of the *M. purpurascens* 7D was confirmed to involve recombination between two parental species. Because of strict uniparental inheritance of chloroplast DNA in most plant species, homologous chloroplast DNA recombination is believed to be absent (Corriveau and Coleman 1988; Nagata 2010; Zhang and

Sodmergen 2003). So far, it has only been reported in hybrids of *Chlamydomonas eugametos* and *C. moewusii* and in the somatic hybrids of *Nicotiana plumbaginifolia* and *N. tabacum* (Lemieux et al. 1981, 1984; Medgyesy et al. 1985). However, mitochondrial DNA homologous recombination has been confirmed in many animals and plants (Burzyński et al. 2003; Ciborowski et al. 2007; Gantenbein et al. 2005; Hoarau et al. 2002; Jaramillo-Correa and Bousquet 2005; Kraytsberg et al. 2004; Städler and Delph 2002; Tsaousis et al. 2005; Ujvari et al. 2007). These chloroplast DNA and mitochondrial DNA recombination events were predominantly discovered in interspecific hybrids, and there are two possible explanations: (1) The frequency of intraspecific homologous recombination in chloroplast or mitochondrial genomes may be much higher than reported, possibly because the absence of diagnostic DNA markers makes it difficult to detect. (2) Molecular recognition systems that result in destruction of chloroplast DNA or mitochondrial DNA may be relaxed during interspecific hybridizations, increasing the frequency of parental leakage (the survival of chloroplast DNA or mitochondrial DNA in sperms or zygotes) in such crosses (Rokas et al. 2003).

Acknowledgments This research was funded by the National High-tech R&D Program (863 Program) of China (2011AA10020903, 2012AA10180104) and the Mendel Biotechnology Inc., USA

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