Genetic analysis of putative triploid *Miscanthus* hybrids and tetraploid *M. sacchariflorus* collected from sympatric populations of Kushima, Japan

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Abstract

Miscanthus ×*giganteus*, which is a triploid hybrid between tetraploid *M. sacchariflorus* and diploid *M. sinensis*, has considerable potential as a bioenergy crop. Currently only one genotype is widely cultivated, increasing its vulnerability to diseases during production. Finding new hybrids is important to broaden genetic resources of *M.* ×*giganteus.* Three putative triploid hybrids were discovered in sympatric population of tetraploid *M. sacchariflorus* and diploid *M. sinensis* in Kushima, Japan. The hybrid nature of the triploids was determined by morphological analysis and sequencing the ribosomal DNA internal transcribed spacer region. The triploids had awns on their florets, which is a common characteristic of diploid *M. sinensis*, and sheath hairs, which is typical of tetraploid *M. sacchariflorus*. All triploids showed heterozygosity in their ribosomal DNA internal transcribed spacer sequences. Based on these results, it is confirmed that the triploids are hybrids and novel genotypes of *M.* ×*giganteus.* Natural crossing between tetraploid *M. sacchariflorus* × diploid *M. sinensis* may also lead to the production of tetraploid hybrids. ITS analysis of tetraploid plants showed that one maternal parent of the triploid hybrids, K-Ogi-1 had heterozygous ITS, which was different to the other analyzed tetraploid *M. sacchariflorus*. Thus, K-Ogi-1 was likely of hybrid origin. These tetraploid hybrids can also be utilized as parents in *M.* ×*giganteus* breeding. Since all hybrids identified in this study had tetraploid *M. sacchariflorus* as maternal parents, collecting and analyzing seeds from tetraploid *M. sacchariflorus* in sympatric areas could be an effective strategy to identify natural *Miscanthus* hybrids that can be used as bioenergy crops.

Introduction

A widespread increase in consumption of easily extractable crude oil, which is a rapidly diminishing resource, has been implicated in helping accelerate global warming [1-3]. Due to these factors, interest in renewable, carbon-neutral sources of energy, such as highly productive feedstock crops and wild plant species, has considerably increased in recent years [1,4,5]. *Miscanthus* ×*giganteus* Greef & Deuter ex Hodkinson & Renvoize, which is a perennial triploid grass native to Japan, exhibits promise as a bioenergy crop because of its high energy-conversion efficiency due to C4 photosynthesis [6], high biomass productivity [7], low fertilizer requirements [8,9], tolerance of low winter temperatures [6,7], and minimal likelihood for invasive spread by seed due to its sterile nature [10]. However, presently only one genotype of *M.* ×*giganteus* is widely cultivated [7], increasing the risk of widespread mortality due to diseases or pests [7,11]. Therefore, finding new genotypes of *M.* ×*giganteus* from natural populations will provide much-needed genetic variation that can be used for bioenergy production.

Currently propagated *M.* ×*giganteus* clones, such as the widely cultivated Illinois clone [12,13] originated from a plant that was introduced to Denmark in 1935 from Yokohama, Japan by a Danish plant collector, Aksel Olsen [14]. The taxon is a natural allotriploid hybrid (3*n*=57) derived from a cross between tetraploid *M. sacchariflorus* (Maxim) Hack. (4*n*=76) and diploid *M. sinensis* Anderss. (2*n*=38) [13,15-18]. Based on chloroplast DNA analysis, *M.* ×*giganteus* has tetraploid *M. sacchariflorus* as its maternal parent [18]. Other natural hybrids between diploid *M. sinensis* and tetraploid *M. sacchariflorus* were discovered in central (Hyogo and Gifu Prefectures) and southern (Kumamoto Prefecture) Japan [19-21]. Honda [19] identified a putative hybrid from the Kuma River Basin in Kumamoto Prefecture, which he named *Miscanthus ogiformis* Honda. *M. ogiformis* exhibited similar aboveground growth as *M. sacchariflorus,* but the spikelets had awns similar to that of *M. sinensis.* Hirayoshi et al. [20] identified two triploid hybrids, which were grown from seeds collected from *M. sinensis* near Gifu, Japan. Their morphological features were apparently similar to that of *M. sacchariflorus,* and their triploid nature was confirmed by cytological analysis. Two natural *Miscanthus* triploids were also collected near Akashi and Sasayama in Hyogo Prefecture, Japan [21]. We postulate that more natural hybrids can be found in overlapping populations of *M. sinensis* and *M.*

sacchariflorus across Japan [22,23]. These natural hybrids can be identified and characterized by morphological and molecular methods.

Recently, triploids were identified from seeds collected from tetraploid *M. sacchariflorus* plants, which were in a sympatric population with diploid *M. sinensis* plants near Kushima, Miyazaki Prefecture, Japan [23]. The nuclear DNA contents of the triploids ranged from 6.7±0.1 to 7.0±0.1 pg·$2C^{-1}$, which were close to that of the Illinois clone of *M. ×giganteus* (7.0±0.1 pg·$2C^{-1}$) [12, 23]. It is hypothesized that the triploids were hybrids between diploid *M. sinensis* and tetraploid *M. sacchariflorus* [23], but the flow cytometry data needs to be further validated by molecular and morphological methods for hybrid detection.

Nuclear ribosomal DNA internal transcribed spacer (ITS) region is widely utilized for detecting hybrid origins of plant taxa [24]. ITS region of plants with hybrid origin is generally homogenized toward one parent type over cycles of sexual reproduction (concerted evolution) [24]. The maternal parents of putative triploid hybrids, tetraploid *M. sacchariflorus,* were suggested to have a hybrid origin between diploid *M. sinensis* and diploid *M. sacchariflorus* [15,18]. Therefore, the ITS sequence of tetraploid *M. sacchariflorus* may be homogenized toward diploid *M. sinensis*. However, *M. ×giganteus* possessed two types of ribosomal DNA ITS regions, one of which corresponds to that of diploid *M. sinensis* and the other that of diploid *M. sacchariflorus*, indicating that ITS sequence of diploid *M. sacchariflorus* is still retained in tetraploid *M. sacchariflorus* [18]. Therefore, we postulated that it is possible to use ITS sequence in determining hybrid nature of putative triploid hybrids.

In addition to determining ITS region of the putative triploid hybrids, the ITS regions of maternal plants were also determined. Tetraploid *M. sacchariflorus* plants from Kushima had higher seed set compared to tetraploid *M. sacchariflorus* in Miyazaki, Tsukuba, Gifu, and Tomakomai sympatric populations [23]. Tetraploid *M. sacchariflorus* usually propagates by spreading rhizomes [23], but high seed set indicates that tetraploid *M. sacchariflorus* plants also propagate through sexual reproduction. Considering that *Miscanthus* genus shows high self-incompatibility [25], the high seed set was not the result of self-fertilization. Therefore, there is a possibility that crossings occur frequently between two tetraploid *M. sacchariflorus* plants or between tetraploid *M. sacchariflorus* and diploid *M. sinensis* in Kushima sympatric populations. Hybridization between diploid *M. sinensis*

var. *condensatus* as mother and tetraploid *M. sacchariflorus* produced not only triploid but also a tetraploid plant that was morphologically similar to tetraploid *M. sacchariflorus* [26]. If tetraploid hybrids occur frequently, tetraploid plants in the population in Kushima may consist of tetraploid hybrids and tetraploid *M. sacchariflorus*, which are genetically distinct from tetraploid *M. sacchariflorus* from Miyazaki, Gifu, Tsukuba, and Tomakomai sympatric populations. Different genotypes of tetraploid plants are valuable parental sources for breeding new *M. ×giganteus.*

The first objective of this study was to confirm the hybrid nature of triploid *Miscanthus* plants by comparing morphological characteristics to diploid *M. sinensis* and tetraploid *M. sacchariflorus*, and by determining their nuclear ribosomal DNA ITS sequences. The second objective was to determine whether there are genetic differences, as reflected in their ITS and chloroplast DNA sequences, between maternal parents of putative triploid hybrids with tetraploid *M. sacchariflorus* from Miyazaki, Gifu, Tsukuba, and Tomakomai sympatric populations.

Materials and Methods

Morphological characterization of putative triploid hybrids

Triploid *Miscanthus* plants, which were labeled Hy-1, Hy-2, and Hy-3, were collected as seed from the inflorescence of tetraploid *M. sacchariflorus*. In the previous study [23] Hy-1, Hy-2, and Hy-3 were labeled as Ogi63, Ogi79, and Ogi80, respectively. Hy-1 was collected as seed from the inflorescence of a tetraploid *M. sacchariflorus* labeled as K-Ogi-1, whereas Hy-2 and Hy-3 were collected from the inflorescence of a tetraploid *M. sacchariflorus* labeled as K-Ogi-2. Two *M. sinensis* plants located adjacent to K-Ogi-1 and K-Ogi-2 were identified as putative pollen parents, and labeled as K-Susuki-1 and K-Susuki-2, respectively. The detailed location of the plants in the sympatric area of Kushima, Miyazaki, Japan is described in Nishiwaki et al. [23]. The Hy-1, Hy-2, and Hy-3 plants, which were morphologically characterized, were propagated from the original genotypes as rhizomes with 2-3 shoots. The plants were established in 10-L pots containing Andisol soil at a research farm adjacent to the University of Miyazaki in April 2011. The

Illinois clone of *M.* ×*giganteus* [12, 13], K-Susuki-1, K-Susuki-2, K-Ogi-1, and K-Ogi-2 were also grown as rhizomes with 2-3 shoots in pots under similar conditions as the triploid plants in spring 2010.

Hodkinson et al. [27] provided morphological keys to identify several species of *Miscanthus*. Two characters, the presence or absence of awns on florets, and the presence or absence of sheath hairs can be used as keys to distinguish diploid *M. sinensis* and tetraploid *M. sacchariflorus* grown in pots. Diploid *M. sinensis* has awns on florets but no sheath hairs, whereas tetraploid *M. sacchariflorus* shows the opposite (i.e., no awns on florets but has sheath hairs). The presence/absence of awns on florets observation was carried out after flowering time in October 2011. The presence/absence of leaf sheaths hair was examined in October 2011. Relatively young and thick stems were chosen for the observation of the presence/absence of leaf sheath hairs.

ITS region sequencing

The ITS sequences of Hy-1, Hy-2, Hy-3, Illinois clone of *M.* ×*giganteus*, maternal parents (K-Ogi-1 and K-Ogi-2), and putative pollen parents (K-Susuki-1 and K-Susuki-2) were sequenced. In addition, the ITS sequences of tetraploid *M. sacchariflorus* and diploid *M. sinensis* from four sympatric populations in Japan (Miyazaki, Gifu, Tsukuba, and Tomakomai) were sequenced. One accession of each species was analyzed for each population. The geographical coordinates of the sympatric populations were described in Nishiwaki et al. [23]. DNA was extracted from leaves using a DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) following the protocol of the manufacturer. The forward and reverse primers were designed following Sun et al. [28] to amplify the ITS regions of each *Miscanthus* taxon. The polymerase chain reaction (PCR) was carried out with the primer set and ExTaq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan). Both reactions consisted of an initial denaturation at 94°C for 3 min; followed by 38 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s), and extension (72°C for 80 s); then a final extension at 72°C for 7 min. Amplification products were confirmed by electrophoresis on 1.0% agarose gel containing ethidium bromide. The electrophoresis was conducted at 100 V for 30 min. PCR products were purified with ExoSAP-IT prior to the sequencing analysis. Sequencing was

performed using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) following the protocol of the manufacturer. Sequence heterogeneity was found within PCR amplification products of Hy-1, Hy-2, and Hy-3. To obtain two different ITS sequences, the PCR amplification products were cloned into the pGEM-T Easy vector (Promega Corp., Tokyo, Japan). Vectors containing DNA fragments were amplified using *Escherichia coli* strain JM109 (Promega Corp.) After overnight culture, plasmids were isolated using High Pure Plasmid Isolation Kit (Roche Applied Science). Plasmids were sequenced using ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

Chloroplast analysis

Four chloroplast DNA regions (*psbC-trnS*, *trnS-trnT*, *trnL-trnF*, and *rpl20-rps12*) of the triploid hybrids, maternal parents (K-Ogi-1 and K-Ogi-2), putative pollen parents (K-Susuki-1 and K-Susuki-2) and *M.* ×*giganteus* were sequenced. DNA was extracted from leaves using a DNeasy Plant Mini Kit (Qiagen). Chloroplast regions were amplified by PCR using specific primer pairs shown in Table 1. The PCR was carried out with the primer set (Table 1) and ExTaq DNA polymerase (TaKaRa Bio). PCR reactions, electrophoresis and sequencing methods were identical to that done for the ITS region analysis.

Sequence data analysis

Sequencing data were aligned using version 2.6 of the SeqScape software (Applied Biosystems) and adjusted manually as necessary. We analyzed a combined data set including all four chloroplast DNA regions. We determined chloroplast DNA haplotypes from nucleotide substitutions and insertions or deletions (indels). Indels were treated as single-mutation events. The ITS sequences were aligned using the same software, and adjusted manually when necessary. The following sequences have been deposited in DNA Data Bank of Japan with ID number in parentheses: *psbC-trnS* of K-Susuki-1 (AB670333), K-Susuki-2 (AB670332), K-Ogi-1 (AB670334); *trnS-trnT* of K-Susuki-1 (AB670340),

K-Susuki-2 (AB670339), K-Ogi-1 (AB670341); *trnL-trnF* of K-Susuki-1 (AB670347), K-Susuki-2 (AB670346), K-Ogi-1 (AB670348); and *rpl20-rps12* of K-Susuki-1 (AB670354), K-Susuki-2 (AB670353), K-Ogi-1 (AB670355).

Results

Comparison of morphological characteristics of the putative triploid hybrids compared to M. ×giganteus*, diploid* M. sinensis*, and tetraploid* M. sacchariflorus

Diploid *M. sinensis* plants did not have hairs on their sheaths, but had awns on their florets (Figure 1A, B). The tetraploid *M. sacchariflorus* plants had hairs on the sheaths, but no awns on their florets (Figure 1A, B). The Illinois clone of *M. ×giganteus* had hairs on their sheaths but no awns on its florets (Figure 1A, B). Hy-1, Hy-2, and Hy-3 possessed hairs on their sheaths as in tetraploid *M. sacchariflorus* (Figure 1A). All putative hybrids had awns on their florets as observed in *M. sinensis* (Figure 1B).

Comparison of ITS sequences between putative triploid hybrids, M. ×giganteus, *diploid* M. sinensis, *and tetraploid* M. sacchariflorus

Direct sequences of ITS regions of Hy-1, Hy-2, and Hy-3 showed that all putative hybrids had heterozygous sequences. Clones of the ITS sequences indicated that Hy-1 had two types of ITS sequences, one matched that of K-Susuki-2 and the other was a typical ITS sequence of tetraploid *M. sacchariflorus* (Table 2). Likewise, the ITS sequences of Hy-2 and Hy-3 were heterozygous. One of the ITS sequences of Hy-2 and Hy-3 matched that of K-Susuki-2 and the other matched that of K-Ogi-2 (Table 2). There were several distinct nucleotide polymorphisms between the ITS sequences of Hy-1, Hy-2, and Hy-3 to that of *M. ×giganteus.* At ITS-177, the genotype of *M. ×giganteus* was ‘G’, whereas the genotypes of the all triploids were ‘A/G’. The genotypes of Hy-2 and Hy-3 were ‘A/G’ at ITS-274, whereas the genotypes of Hy-1 and *M. ×giganteus* were ‘A’ at ITS-274. K-Ogi-1 (the

maternal parent of Hy-1) showed heterozygosity in the ITS sequence at ITS-177 ('A/G'), ITS-291 ('A/G'), ITS-331 ('C/T'), ITS-337 ('C/T'), and ITS-525 ('GT/AGGG') (Table 2). K-Ogi-2 also showed heterozygosity at ITS-274 ('A/G') and ITS-285 ('C/T') (Table 2). In contrast to K-Ogi-1 and K-Ogi-2, tetraploid *M. sacchariflorus* from Tomakomai, Tsukuba, Gifu and Miyazaki were homozygous in their ITS sequences. The sequence was specific to tetraploid *M. sacchariflorus* and can be differentiated with the ITS sequences of diploid *M. sinensis* based on polymorphisms at ITS-291, ITS-331, ITS-337, and ITS-525 (Table 2). The genotypes of diploid *M. sinensis* accessions from Kushima, Miyazaki, Gifu, Tsukuba and Tomakomai at ITS-291, ITS-331, ITS-337, and ITS-525 were 'G', 'T', 'T', and 'GT' respectively; whereas the genotypes of tetraploid *M. sacchariflorus* accessions from Miyazaki, Gifu, Tsukuba and Tomakomai were 'A', 'C', 'C', and 'AGGG' respectively.

Chloroplast DNA sequence of K-Ogi-1, K-Ogi-2, tetraploid M. sacchariflorus *and diploid* M. sinensis *of Kushima*

Chloroplast DNA sequence results are shown in Table 3. There are indel polymorphisms after the position 669-bp in the region of *trnL–trnF*, but they were excluded from further analysis because of alignment complexity. Diploid *M. sinensis* (K-Susuki-1, K-Susuki-2) and tetraploid *M. sacchariflorus* (K-Ogi-1 and K-Ogi-2) could be distinguished by nucleotide substitutions at position 57-bp in *trnS-trnT* region, 271-bp in *trnL-trnF* region, and 671-bp in *rpl20-rps12* region (Table 3). In addition, tetraploid *M. sacchariflorus* had 6 bp inserts at position 212-bp in *trnS-trnT* region, and 17 bp deletion at position 1002-bp in the *trnS-trnT* region. The chloroplast DNA sequences of tetraploid *M. sacchariflorus* from Miyazaki, Gifu, Tsukuba, and Tomakomai sympatric populations also showed 6 bp insert at position 212-bp and 17 bp deletion at position 1002-bp in *trnS-trnT* region as in K-Ogi-1 and K-Ogi-2 (data not shown). Moreover, the tetraploid *M. sacchariflorus* plants also had 'C' at position 57-bp of *trnS-trnT* region, 'G' at position 271-bp in *trnL-trnF* region, and 'A' at position 671-bp in *rpl20-rps12* region as in as in K-Ogi-1 and K-Ogi-2 (data not shown). Meanwhile, the triploid hybrids and *M.* ×*giganteus* had chloroplast DNA typical of tetraploid *M. sacchariflorus*.

Discussion

The morphological characteristics of the triploid accessions Hy-2 and Hy-3 appeared to be a combination of the characteristics of diploid *M. sinensis* and tetraploid *M. sacchariflorus*. The ITS regions of Hy-2 and Hy-3 were a combination of the sequences of maternal plant K-Ogi-2 with putative pollen parent K-Susuki-2. Based on these results and the karyotype analysis results reported in Nishiwaki et al. [23], we conclude that Hy-2 and Hy-3 are triploid hybrids of diploid *M. sinensis* and tetraploid *M. sacchariflorus*, and can be classified as *M.* ×*giganteus*.

The ITS sequences of Hy-1 were a combination between K-Ogi-1 and K-Susuki-2, but the maternal parent K-Ogi-1 also had heterozygous ITS sequences, which were identical to that of Hy-1. Consequently, the hybrid origin of Hy-1 cannot be determined based on ITS sequence data alone. Additional studies using molecular markers such as SSRs, EST-SSRs, or SNPs will confirm the present results. Hy-1 had awns on its florets, which is typical of *M. sinensis*, and had hairs on its leaf sheaths as in *M. sacchariflorus* (Table 3). In addition, the DNA content of Hy-1 was similar to that of *M.* ×*giganteus* [23]_ Based on these results, we also conclude that Hy-1 can be classified as *M.* ×*giganteus*. Hy-1, Hy-2 and Hy-3 possessed *M. sacchariflorus* type of chloroplast DNA similar to *M.* ×*giganteus*. Along with morphological differences, Hy-1, Hy-2, and Hy-3 may vary to the widely cultivated *M.* ×*giganteus* in resistance to diseases or pests [29-32], lignin and cellulose composition [33], or mineral content [34-35]. Newly found triploid hybrids can serve as additional genetic sources of the widely cultivated *M.* ×*giganteus* clone.

Both K-Ogi-1 and K-Ogi-2 showed heterozygosity in their ITS sequences, but only the ITS sequence of K-Ogi-1 showed a combination between tetraploid *M. sacchariflorus* and diploid *M. sinensis* (Table 2). In addition, tetraploid *M. sacchariflorus* from Miyazaki, Gifu, Tsukuba, and Tomakomai possessed homozygous ITS sequences. The ITS sequence of tetraploid *M. sacchariflorus* can be distinguished to that of diploid *M. sinensis* based on nucleotide ITS-291, ITS-331, ITS-337, and ITS-525 (Table 2). Although tetraploid *M. sacchariflorus* is suggested to have a hybrid origin between diploid *M. sinensis* and diploid *M. sacchariflorus* [15, 36], it seems that only one ITS sequence retained in tetraploid *M. sacchariflorus*. Based on these results, K-Ogi-1 may be derived from a recent crossing event involving a tetraploid *M. sacchariflorus* and a diploid *M. sinensis*. In addition

to ITS sequence, the chloroplast DNA type of K-Ogi-1 was also determined. K-Ogi-1 had chloroplast DNA typical of tetraploid *M. sacchariflorus*, indicating that the maternal parent of K-Ogi-1 was possibly a tetraploid *M. sacchariflorus*, and the pollen parent was diploid *M. sinensis.* More tetraploid hybrids like K-Ogi-1 may be found in sympatric population of tetraploid *M. sacchariflorus* and diploid *M. sinensis*. Indeed, it was reported that artificial hybridization between diploid *M. sinensis* var. *condensatus* and tetraploid *M. sacchariflorus* produced tetraploid hybrids besides triploid hybrids [26]. Because of the difference in genome composition to tetraploid *M. sacchariflorus,* these tetraploid hybrids can also be utilized as parents in *M.* ×*giganteus* breeding programs.

In this study, three triploids were confirmed to be hybrids between tetraploid *M. sacchariflorus* and diploid *M. sinensis.* In addition, a maternal parent of one of the triploid hybrids also had hybrid origin. Finding both triploid hybrids and a tetraploid hybrid in Kushima sympatric population supported the suggestion by Nishiwaki et al. [23] that hybridization frequently occurs in the sympatric areas where tetraploid *M. sacchariflorus* shows high seed set. Further investigation in such sympatric areas may reveal more natural hybrids between tetraploid *M. sacchariflorus* and diploid *M. sinensis*. Based on previous result, hybrids may be found in Gifu sympatric population, where the seed set of tetraploid *M. sacchariflorus* was relatively high [23]. Indeed, triploid hybrids were identified in populations in Gifu in 1957 [20]. Gifu is also interesting because it is located northern of Kushima and has a colder climate, therefore the *Miscanthus* plants may have different flowering times, growth velocity, or higher tolerance to cold than the triploid hybrids identified in Kushima. Since hybrids identified in this study had tetraploid *M. sacchariflorus* as the maternal parent, collecting and analyzing seeds from tetraploid *M. sacchariflorus* in such areas is a good strategy to identify natural *Miscanthus* hybrids that can be used as bioenergy crops.

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Table 1 – Primer sets used to amplify chloroplast DNA regions of putative triploid hybrids, *M.* ×*giganteus*, diploid *M. sinensis*, and tetraploid *M. sacchariflorus*

Chloroplast DNA region	Direction	Primer sequence	Reference
trnL - trnF	Forward	5'-CGAAATCGGTAGACGCTACG-3'	[37]
	Reverse	5'-ATTTGAACTGGTGACACGAG-3'	
psbC - trnS	Forward	5'-GGTCGTGACCAAGAAACCAC-3'	[38]
	Reverse	5'-GGTTCGAATCCCTCTCTCTC-3'	
trnS - trnT	Forward	5'-CGAGGGTTCGAATCCCTCTC-3'	[38]
	Reverse	5'-AGAGCATCGCATTTGTAATG-3'	
rpl20 -rps12	Forward	5'-TTTGTTCTACGTCTCCGAGC-3'	[39]
	Reverse	5'-GTCGAGGAACATGTACTAGG-3'	

Table 2 – Polymorphisms in ITS region sequences of the putative triploid hybrids, *M.* ×*giganteus*, diploid *M. sinensis*, and tetraploid *M. sacchariflorus*

Accession	Origin	Copy	Position in ITS sequence						
			177	274	285	291	331	337	525
Putative hybrids									
Hy-1	Kushima	1	A	A	C	G	T	T	GT
		2	G	A	C	A	C	C	AGGG
Hy-2	Kushima	1	A	A	C	G	T	T	GT
		2	G	G	C	A	C	C	AGGG
Hy-3	Kushima	1	A	A	C	G	T	T	GT
		2	G	G	T	A	C	C	AGGG
M. ×*giganteus*									
M. ×*giganteus*	Illinois		G	A	C	A/G	C/T	C/T	GT/AGGG
M. sinensis									

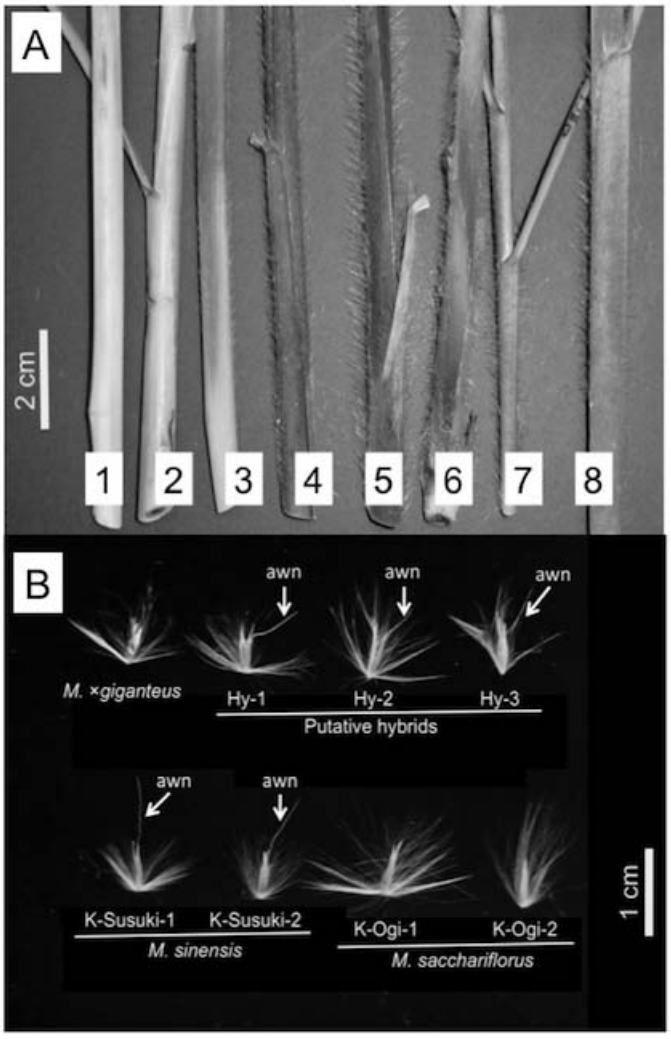
K-Susuki-1	Kushima	A	A	T	G	T	T	GT
K-Susuki-2	Kushima	A	A	C	G	T	T	GT
1062	Tomakomai	G	A	C	G	T	T	GT
1145	Tsukuba	G	A	C	G	T	T	GT
1042	Gifu	G	A	C	G	T	T	GT
1001	Miyazaki	A/G	A	C	G	T	T	GT
M. sacchariflorus								
K-Ogi-1	Kushima	A/G	A	C	A/G	C/T	C/T	GT/AGGG
K-Ogi-2	Kushima	G	A/G	C/T	A	C	C	AGGG
42	Tomakomai	G	G	C	A	C	C	AGGG
89	Tsukuba	G	A/G	C	A	C	C	AGGG
24	Gifu	G	A	C	A	C	C	AGGG
1	Miyazaki	G	G	C	A	C	C	AGGG

Table 3 – Chloroplast type of putative triploid hybrids, Illinois clone of *M.* ×*giganteus*, diploid *M. sinensis* and tetraploid *M. sacchariflorus* collected from Kushima sympatric population

Accession	*psbC-trnS*				*trnS-trnT*					*trnL-trnF*[a)]			*rpl20-rps12*	
	183	216	441	1158	57	212	394	622	1002	271	389	394	172	671
Putative hybrids														
Hy-1, Hy-2, Hy-3	A	T	C	G	C	6bp	G	T	-	G	C	T	G	A
M. ×*giganteus*														
M. ×*giganteus*	A	T	C	G	C	6bp	G	T	-	G	C	T	G	A
M. sinensis														
K-Susuki-1	A	T	C	A	A	-	A	T	17bp[2)]	T	A	A	G	C
K-Susuki-2	A	T	C	A	A	-	A	T	17bp	T	C	A	T	C
M. sacchariflorus														
K-Ogi-1	A	T	C	G	C	6bp[3)]	G	T	-	G	C	T	G	A
K-Ogi-2	A	T	C	G	C	6bp	G	T	-	G	C	T	G	A

a) The indel polymorphisms at position 669 bp in *trnL-trnF* region was excluded from further analysis because of the alignment complexity. 17 bp insertion: AGTAACACAAAAAATGG, 6 bp insertion: GGGGAA

Figure 1. Three putative triploid hybrids (Hy-1, Hy-2, and Hy-3), Illinois clone of *M.* ×*giganteus*, tetraploid *M. sacchariflorus* (K-Ogi-1 and K-Ogi-2) and diploid *M. sinensis* (K-Susuki-1 and K-Susuki-2) were examined for the presence or absence of leaf sheath hairs and awns on florets. A) Leaf sheaths of putative triploid hybrids (Hy-1, Hy-2, and Hy-3), *M.* ×*giganteus*, tetraploid *M. sacchariflorus* (K-Ogi-1 and K-Ogi-2) and diploid *M. sinensis* (K-Susuki-1 and K-Susuki-2). Number 1, 2, 3, 4, 5, 6, 7, and 8 were K-Susuki-1, K-Susuki-2, *M.* ×*giganteus*, Hy-1, Hy-2, Hy-3, K-Ogi-1, and K-Ogi-2, respectively. K-Susuki-1 and K-Susuki-2 do not have leaf sheath hair whereas other plants have. B) Awns on florets of putative triploid hybrids (Hy-1, Hy-2, and Hy-3), *M.* ×*giganteus*, tetraploid *M. sacchariflorus* (K-Ogi-1 and K-Ogi-2), and diploid *M. sinensis* (K-Susuki-1 and K-Susuki-2).


A
2 cm
1
2
3
4
5
6
7
8
B
awn
awn
awn
M. ×giganteus
Hy-1
Hy-2
Hy-3
Putative hybrids
awn
awn
K-Susuki-1
K-Susuki-2
M. sinensis
K-Ogi-1
K-Ogi-2
M. sacchariflorus
1 cm