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Discovery of Natural Interspecific Hybrids Between *Miscanthus Sacchariflorus* and *Miscanthus Sinensis* in Southern Japan: Morphological Characterization, Genetic Structure, and Origin

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Abstract Natural *Miscanthus* grasses are useful for improving biomass production. We found a population of putative triploid interspecific hybrids between *Miscanthus sacchariflorus* and *Miscanthus sinensis* in southern Kyushu, Japan. This study aims to investigate its morphological variation, genetic structure, and origin. *Miscanthus* plants were collected from 114 points, mainly beside a river along a distance of 2.8 km in the Tashiro–Fumoto area. They resembled *M. sacchariflorus* but showed morphology intermediate between the two species. They had a nuclear DNA content corresponding to that of a hybrid between tetraploid *M. sacchariflorus* and diploid *M. sinensis*, and had species-specific alleles from both species revealed by DNA marker analysis. This indicates that the plants are triploid hybrids between *M. sacchariflorus* and *M. sinensis*. Genotyping using simple sequence repeat markers revealed only four genotypes among the hybrid population, of which two accounted for most plants. The genotypes showed mostly discrete geographical distributions. The two major genotypes showed contrasting phenotypes in pollen viability and in frequency of awns in florets. Some seeds collected from the population germinated and the seedlings showed a wide range of nuclear DNA content from diploid to tetraploid. In this area, many *M. sinensis* plants also grew, but we could not find *M. sacchariflorus*. The hybrid *Miscanthus* might be selected due to its improved adaptability introduced from *M. sinensis*. Furthermore, genetic and phenotypic characterization suggests the polyphyletic origin and clonal propagation of this population. Such partially fertile hybrids could be interesting for the improvement of *Miscanthus* as a biomass crop.

Keywords Biomass crop · Fertility · Interspecific hybrid · *Miscanthus sacchariflorus* · *Miscanthus sinensis*

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Introduction

The genus *Miscanthus* Andersson consists of approximately 12 species of C_4 perennial grasses that originate in a region ranging from East Asia to the Pacific islands [1]. *Miscanthus sacchariflorus* (Maxim.) Bentham and *Miscanthus sinensis* Andersson are the predominant species in Japan. In comparison to *M. sinensis*, *Miscanthus sacchariflorus* prefers wetter soil conditions, such as river banks, but their ranges overlap, sometimes in sympatric populations [2]. In Japan, *M. sinensis* is generally diploid and *M. sacchariflorus* is tetraploid [3], but diploid *M. sacchariflorus* grows in China and Korea [4, 5].

Miscanthus grasses are a popular focus of biomass production in temperate regions as a source of renewable energy on account of their high productivity, low fertilizer requirement, and relatively wide adaptability [1]. In particular, *Miscanthus* ×*giganteus* Greef & Deuter ex Hodk. & Renvoize is a promising crop in Europe and the USA [1, 6, 7]. Cytogenetic and molecular analyses has shown that *M.* ×*giganteus* is an interspecific triploid hybrid between tetraploid *M. sacchariflorus* and diploid *M. sinensis* [8, 9]. It shows remarkably high

productivity due to heterosis. Its sterility, due to its triploid nature [10, 11], is advantageous for limiting the risk of spread in non-native regions. One extensively studied genotype was exported from Yokohama, Japan in the 1930s by the Danish botanist Aksel Olsen [12, 13]. To expand the genetic variation, breeders have purposely crossed *M. sacchariflorus* and *M. sinensis* [1, 14].

Naturally growing interspecific hybrids are also useful for the acquisition of breeding materials. In sympatric population areas, natural interspecific hybrids may arise if the flowering periods overlap [2]. More than half a century ago, studies reported natural putative triploid hybrids between *M. sacchariflorus* and *M. sinensis* in central to southern Japan. A putative triploid hybrid, *Miscanthus ×ogiformis*, was collected in Kumamoto prefecture in Kyushu (southern Japan) and taxonomically named by Honda [15]. Adati [16] found two triploid *Miscanthus* plants in Hyogo prefecture (western Japan), and Hirayoshi et al. [3] identified triploid plants grown from caryopses collected from *M. sinensis* in Gifu prefecture (central Japan).

Since the importance of *Miscanthus* as a biomass crop has grown and molecular techniques such as DNA fingerprinting have become available, the identification of further triploid interspecific hybrids has been reported again in East Asia: Nishiwaki and colleagues [2, 9] used cytological and molecular techniques to identify three interspecific triploid plants grown from caryopses collected from *M. sacchariflorus* in a sympatric population in Miyazaki prefecture, Japan. Moon et al. [4] identified a triploid *Miscanthus* plant with a nuclear DNA content similar to that of *M. ×giganteus* in Chungcheongnam-do, Korea. Ibaragi et al. [17] reexamined plants intermediate in morphology between *M. sacchariflorus* and *M. sinensis* that were previously classified as *M. sinensis* Andersson var. *sunanensis* Y. N. Lee in Korea, and concluded that this taxon should be treated as *M. ×ogiformis* (not *M. ×giganteus*, which is distinguished from *M. ×ogiformis* by the absence of awns in florets). In China, 11 accessions of interspecific hybrids between *M. sacchariflorus* and *M. sinensis* were identified using genome-wide genotyping techniques [18]. In addition to triploid hybrids (*M. ×giganteus* or *M. ×ogiformis*), natural diploid hybrids, previously classified as *Miscanthus oligostachyus* 'Purpurascens', were confirmed using cytological and molecular methods in China [19, 20]. Dwiyanti et al. [9] proposed the existence of a tetraploid hybrid between *M. sacchariflorus* and *M. sinensis* in Japan.

Hybridization and introgression in plants are thought to contribute to range expansion and adaptation in new environments by increasing genetic variability in hybridizing populations through new gene combinations, changes in polyploidy levels, or both [21, 22]. This hypothesis could be tested by phenotypic and genetic characterization of the natural hybrid population, in addition to the investigation of the habitats and growth environments [e.g., 23]. However, for *Miscanthus* hybrids, no natural spontaneous populations have been reported.

In this study, we found putative interspecific hybrids of triploid *Miscanthus* in the Tashiro–Fumoto area of southern Kyushu, Japan; this area lies at the southern limit of the range of *M. sacchariflorus*. This paper firstly aims to describe this putative hybrid population at the landscape scale using morphological, cytological, and molecular genetic methods. Secondly, we propose an explanation of the process of emergence and dispersal of this triploid *Miscanthus* hybrid population.

Materials and Methods

Investigation Site

We originally collected one *Miscanthus* plant in the Tashiro–Fumoto area (31.20°N, 130.85°E, 150–180 m a.s.l.), a small basin along the Fumoto River in Kinko-cho, Kimotsuki-gun, Kagoshima prefecture, Kyushu, Japan (Fig. 1a, b). The Fumoto River runs from east to west in the Tashiro–Fumoto area and is a tributary of Ogawa River, which further flows ca. 9 km into a sea estuary (Fig. 1b, c). The collected plant looked like *M. sacchariflorus*, but showed some intermediate morphology between *M. sacchariflorus* and *M. sinensis*. So we investigated the *Miscanthus* population in this area.

Plant Materials and Measurements

We sampled putative hybrid plants and surveyed the distribution of the population from 114 sites mainly along the Fumoto River over a range of 2.8 km on 31 October 2012 and 3–4 October 2013. Sampling was performed at at least one site in each canopy we found. Leaf samples from 114 sites were used for flow cytometry assay and DNA analysis. One or two panicles were sampled per site. Panicles sampled from 13 out of 114 sites in 2012 were used to evaluate seed fertility (seed-set and germination rates). Flowering panicles from 39 out of 114 sites sampled in 2013 were used to evaluate pollen viability. Panicles from 29 out of 114 sites sampled in 2013 were used to count awned florets. Awns were counted in 30 florets on each of three rachis branches from one panicle. Plant length (length of a straightened shoot from the ground level to the top) and panicle length (first branch on the main axis to the top) were measured for representative shoots at nine sites. Stems and rhizomes extracted from soil were observed for representative plants. Twenty-three *Miscanthus sinensis* plants from this area were also analyzed. Ten *Miscanthus sacchariflorus* accessions collected in southern Kyushu ('Uchinoura,' 'Takayamagawa,' 'Kimotsukigawa,' 'JM0126,' 'Hirosegawa,' 'Gotandagawa,' 'Sendaigawa' and 'Kumagawa') and other regions in Japan ('Morioka' and 'NARCH-OGI-62'), and one *M. ×giganteus* clone 'Illinois', were also used for each analysis.

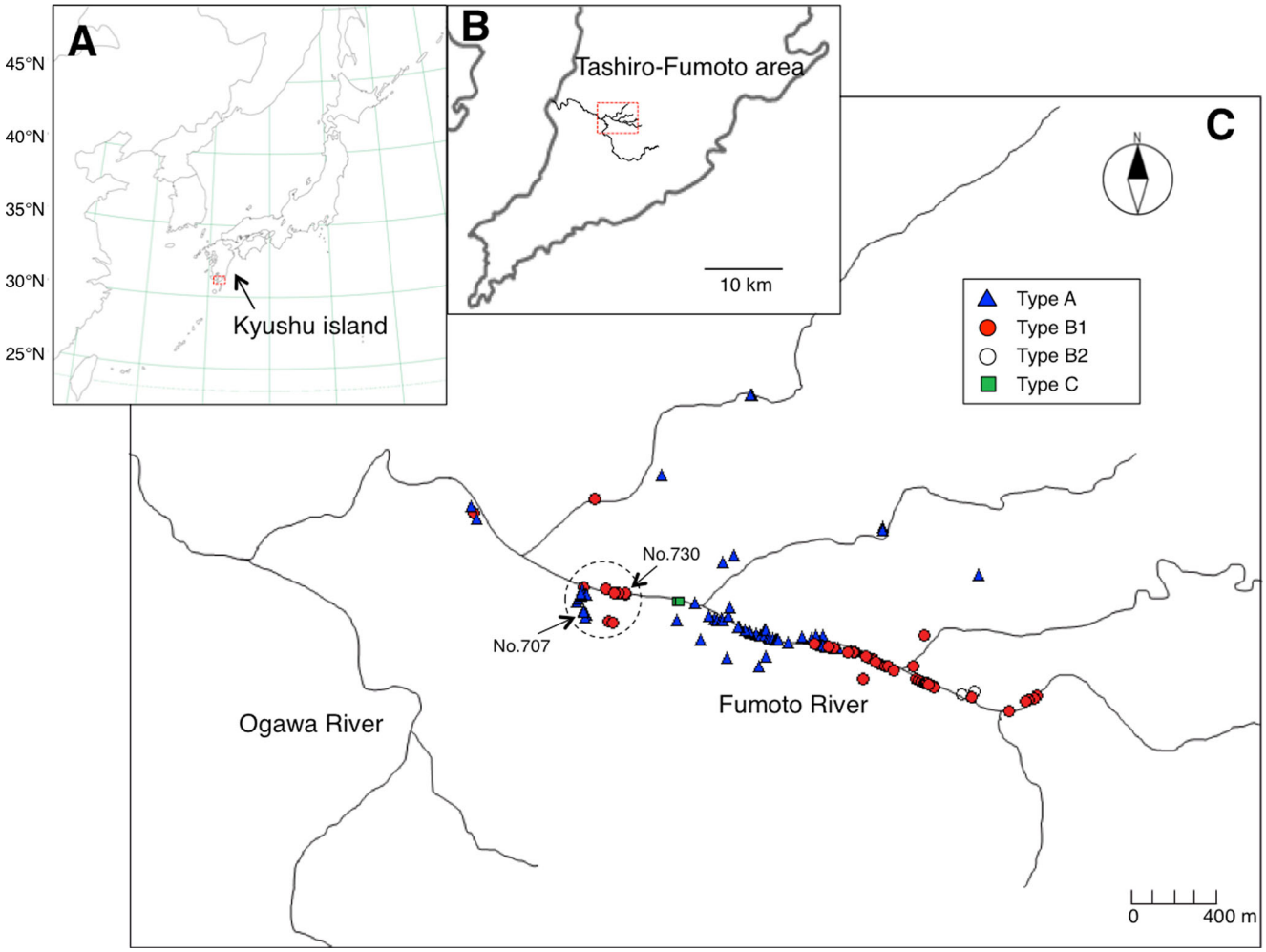

Fig. 1 Geographical distribution of putative triploid hybrids between *M. sacchariflorus* and *M. sinensis*, in the Tashiro–Fumoto area, southern Kyushu, Japan. Symbols mark locations of plants sampled; ▲ (*blue*), type A; ● (*red*), type B1; ○, type B2; ■ (*green*), type C. Genotypes were characterized by using 24 simple sequence repeat markers. *Dashed circle* indicates samples used for seed-set rate and germination tests. Two representative plants nos. 707 and 730 used for chromosome counting are indicated by *arrows*

Estimation of Nuclear DNA Content and Counting of Chromosomes

The relative nuclear DNA content was measured by flow cytometry assay. One tall fescue (*Festuca arundinacea* Schreb.) genotype was used as an internal standard. Nuclei prepared from chopped leaves were stained with 4′,6-diamidino-2-phenylindole (DAPI) fluorochrome by using a CyStain UV precise P reagent kit (Partec GmbH, Münster, Germany), and the filtered solution was analyzed with a Particle Analysing System (Partec). Each sample was assayed twice. Chromosome spreads were prepared by the enzymatic maceration/air-drying method [24, 25]. DAPI-stained chromosomes in root-tip squashes of two plants with two representative different genotypes revealed by DNA analysis (nos. 707 and 730, indicated in Fig. 1c) grown in pots in a glasshouse were counted under a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

Genomic DNA Marker Assay and Clustering Analysis

To reveal the degree of genetic uniformity within the putative hybrid population, we examined the extent of genomic DNA polymorphisms among plants by using SSR markers. Genomic DNA was extracted from leaves by the cetyltrimethylammonium bromide (CTAB) method [26]. Table S1 lists the primer sequences of 7 intron-flanking markers used to distinguish *M. sacchariflorus* and *M. sinensis* [27] and 24 simple sequence repeat (SSR) markers used to evaluate genetic polymorphisms for phylogenetic analysis [28, 29]. Six random genotypes were used to select SSR primers showing polymorphisms in putative hybrids. In SSR marker analysis, in addition to putative hybrids, six *M. sinensis*, six *M. sacchariflorus*, and one *M.* ×*giganteus* plants were genotyped. The polymerase chain reaction (PCR) was performed as described [27]. Products amplified

with intron-flanking primers were loaded into 2 % or 4 % agarose gels in TAE (Tris, acetic acid, EDTA) buffer, and those amplified with SSR primers were loaded into 6 % acrylamide gels in TBE (Tris, borate, EDTA) buffer for electrophoresis. Following staining with ethidium bromide, the banding patterns were photographed under ultraviolet light. Reproducibility of polymorphisms among genotypes of putative *Miscanthus* hybrids was confirmed using one representative sample from each type. Genetic distance matrixes based on SSR polymorphisms were calculated using Nei's index [30]. Clustering was performed by the unweighted pair group method, using the arithmetic mean procedure in R software v. 3.0.2 [31].

Chloroplast DNA Analysis

Four chloroplast DNA regions previously sequenced in some *Miscanthus* genotypes were analyzed; namely, *psbC-trnS*, *trnS-trnT*, *trnL-trnF*, and *rpl20-rps12* [9, 32]. DNA was extracted from 14 putative *Miscanthus* hybrids, six *M. sinensis* plants from Tashiro–Fumoto, and ten *M. sacchariflorus* accessions from other regions of Japan and was used as a PCR template. PCR reaction mixtures (10 μl) contained ca. 100 ng of genomic DNA, 0.2 units of AmpliTaq 360 polymerase (Applied Biosystems), 1× PCR buffer (Applied Biosystems), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, and forward and reverse primers (0.5 mM each) as described by Dwiyanti et al. [9] and Shimono et al. [32]. The cycling regime for the PCR amplification consisted of an initial denaturation step of 2 min at 95 °C; 32 cycles of 30 s at 95 °C, 30 s at 56 °C and 1 min 20 s at 72 °C; and a final extension step of 7 min at 72 °C. PCR products were cleaned up using Exo-SAP IT reagent (Affymetrix, Santa Clara, CA, USA) and directly sequenced using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). All sequences were deposited in the DDBJ database (accession nos. LC060067 to LC060146). Multiple alignment was performed using DNASIS v. 3.0 software (Hitachi Software Engineering, Tokyo, Japan).

Evaluation of Pollen Viability and Seed Fertility

Pollen viability of 39 samples of putative hybrids was investigated. Two lots of pollen grains from five to ten florets each per individual were stained with 2 % acetocarmine solution [33]. Fully stained and unstained grains were counted under a microscope.

Seed-set and germination rates of 13 putative hybrids and 3 *M. sinensis* plants collected in the western part of the Tashiro–Fumoto area were evaluated (Fig. 1c). To estimate the seed-set rate, we used an X-ray inspection apparatus (C-60 TV-PbO-1; Softex, Ebina, Japan) to count filled and empty seeds in three lots of about 100 seeds each per individual. Before the germination test, seeds were maintained at 4 °C over a period of 4 months. Seeds (500–5,000; including empty ones) were placed on wet filter paper and incubated for 4 weeks at 25/15 °C under a daylength of 14 h. The germination ratio (%) was estimated as:

$$\text{No.germinated seeds}/(\text{total No. tested seeds} \times \text{seed-set rate}) \times 100$$

Statistical Analysis

Statistical analyses (ANOVA, Tukey's and Wilcoxon's tests) were performed in JMP software v. 9 (SAS Institute, Cary, NC, USA).

Results

Identification of a Putative Hybrid *Miscanthus* Population in the Tashiro–Fumoto Area of Japan

Putative hybrid *Miscanthus* plants were distributed mainly alongside the Fumoto River over a range of 2.8 km (Fig. 1c). As well as on river banks, some plants grew along the edges and footpaths of fields and on roadsides near the river. Some plants were found around tributaries, up to 1.4 km upstream of their confluence with the river (Fig. 1c). *Miscanthus sinensis* also grew naturally in this area. We could not find any putative hybrid plants in the Ogawa River basin.

The putative hybrids had elongated rhizomes, and some plants formed continuous stands up to 50 m long alongside the river or fields (Fig. 2a, b). Stems grew vertically (Fig. 2b), with buds at the nodes like *M. sacchariflorus* (Fig. 2c, d). The length of the plants on riverbanks ranged from 2.5 to 3.0 m at flowering stage, with panicles measuring 40 to 55 cm. Spikelets had long white callus hairs and either short or no awns on the lemmata (Fig. 2e, f).

The mean fluorescence level of DAPI-stained nuclear DNA of the putative hybrids from 114 sites was 0.396± 0.008 relative to that of *F. arundinacea*. It was 1.30±0.03 times the mean fluorescence level of 20 genotypes of *M. sinensis* (0.305±0.011) growing in this area, almost the same as that of *M.* ×*giganteus* 'Illinois' (1.32 times; Table 1). The fluorescence level of DAPI-stained nuclear DNA of a tetraploid *M. sacchariflorus* strain 'Uchinoura' was 1.57 times that of *M. sinensis*. The chromosome number of two putative hybrids sampled was $2n=57$ (Fig. 3a, b), three times the basic number in *Miscanthus* ($x = 19$). Lastly, primer pairs, which generate specific fragments to *M. sacchariflorus* or *M. sinensis*, amplified five fragments specific to *M. sacchariflorus* and three specific to *M. sinensis* in all 114 samples (Fig. 4).

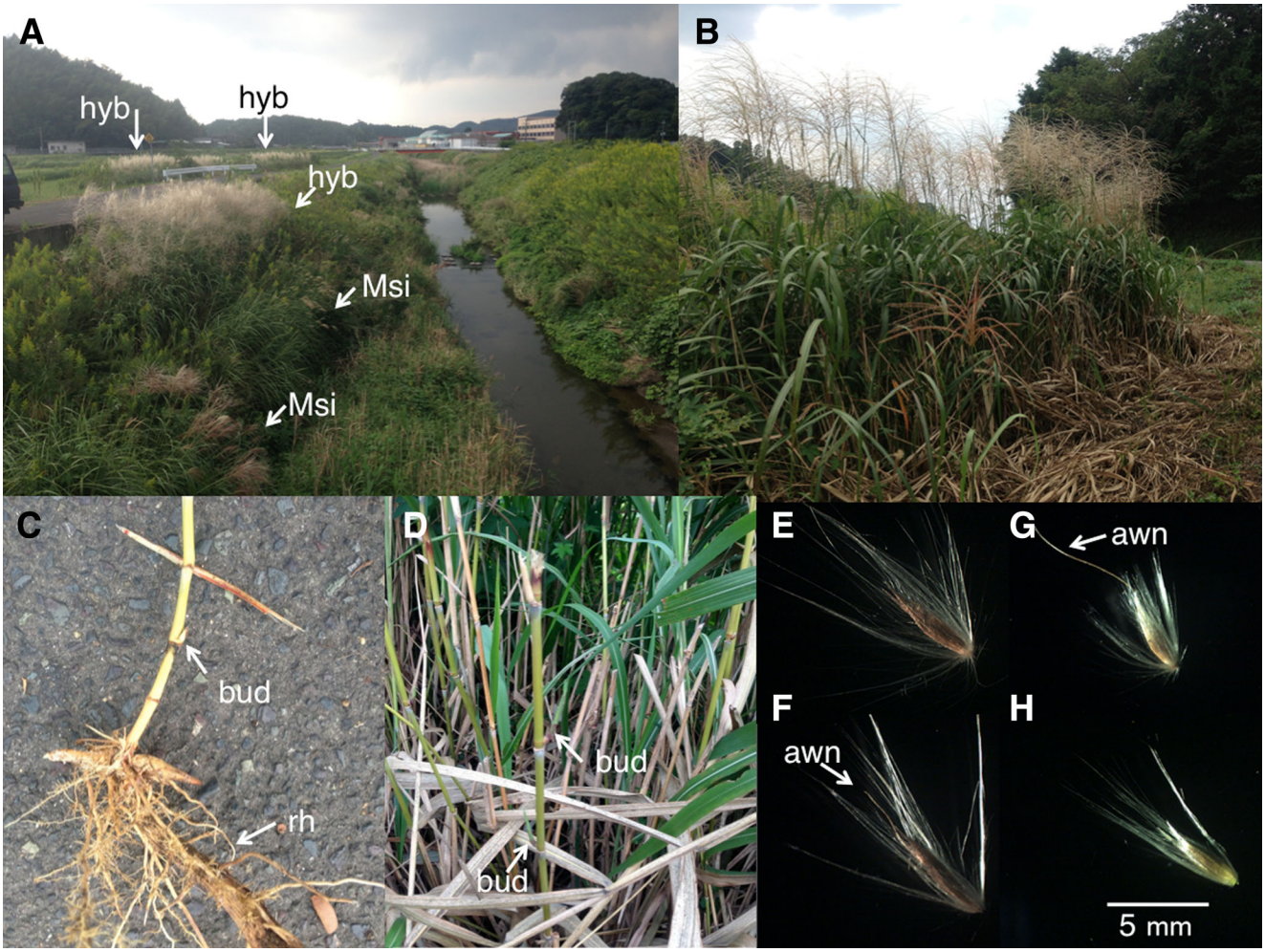

Fig. 2 Morphology of putative triploid hybrids between *M. sacchariflorus* and *M. sinensis* found in the Tashiro–Fumoto area. **a** *Miscanthus* plants growing along Fumoto River (*hyb*, putative triploid hybrid; *Msi*, *M. sinensis*). **b** Putative hybrid plants growing at the edge of a field. **c** Rhizomes (*rh*). **d** Buds at nodes. **e** Awnless spikelet of a putative hybrid. **f** Awned spikelet of a putative hybrid. **g** Spikelet of *M. sinensis*. **h** Spikelet of *M.* ×giganteus 'Illinois'

All these results indicate that all tested plants are triploid hybrids between *M. sacchariflorus* and *M. sinensis*.

Genetic characterization of a putative hybrid *Miscanthus* population in the Tashiro–Fumoto area

Genotyping of the 114 samples using 24 polymorphic SSR markers revealed 94 polymorphic fragments among all 135 fragments. Only four genotypes were evident: A (n=63), B1 (n=47), B2 (n=2) and C (n=2). Types B1 and B2 were distinguished by only one fragment specific to each type.

Clustering analysis of the putative hybrids and other *Miscanthus* accessions revealed three major clusters: *M. sinensis*, *M. sacchariflorus* and putative hybrid *Miscanthus* from Tashiro–Fumoto (Fig. 5). *Miscanthus* ×*giganteus* was clustered with *M. sacchariflorus* (Fig. 5). The putative hybrid

Table 1 Relative fluorescence levels of DAPI-stained nuclei of putative hybrid *Miscanthus* from the Tashiro–Fumoto area and other plants

Tested plants	No. of plants	Fluorescence level relative to	
		Festuca arundinacea	*M.sinensis*
Putative triploid hybrid *Miscanthus* in Tashiro–Fumoto	114	0.396 (SD 0.008)	1.30
M. sinensis in Tashiro–Fumoto	20	0.305 (SD 0.011)	1.00
M. sacchariflorus 'Uchinoura'	1	0.477	1.57
M. ×*giganteus* 'Illinois'	1	0.403	1.32

SD, standard deviation.

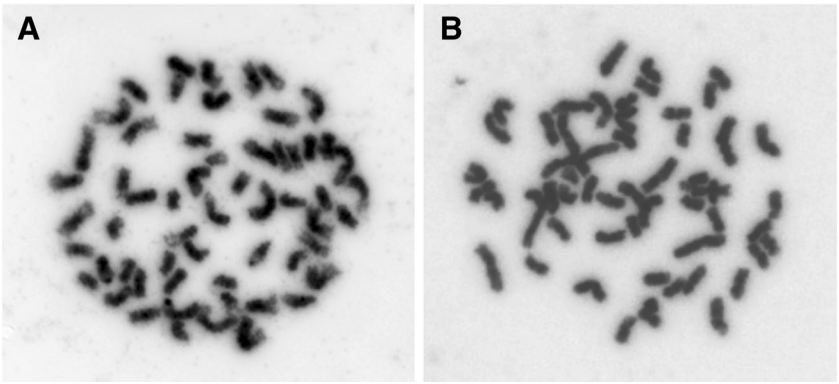

Fig. 3 Fluorescence microscopy of DAPI-stained chromosomes of putative triploid hybrids between *M. sacchariflorus* and *M. sinensis* in Tashiro–Fumoto , plants no. 707 (type A) and no. 730 (type B1). Genotypes were characterized by using 24 simple sequence repeat markers

cluster was genetically closer to *M. sacchariflorus* than to *M. sinensis*. Genetic distances among A, C and B1 + B2 were similar to those among *M. sinensis* from Tashiro–Fumoto and among *M. sacchariflorus* from southern Kyushu (Fig. 5).

Regarding the geographical distribution of the four types of genotypes, type A plants were distributed mainly in the center of the range along a 0.8-km stretch of the Fumoto River (Fig. 1c). A cluster of type B plants was distributed mainly in the eastern part of the range along a 1-km stretch of the river. The two type B2 plants grew 60 m apart. The two plants of type C grew 12 m apart within a continuous band of vegetation at the edge of the type A area. Types A and B1 plants also grew sympatrically in the western part of the range along the river banks or the edges of fields and roads.

Six randomly selected plants of type A and six of type B1 had the same haplotype of four chloroplast DNA regions

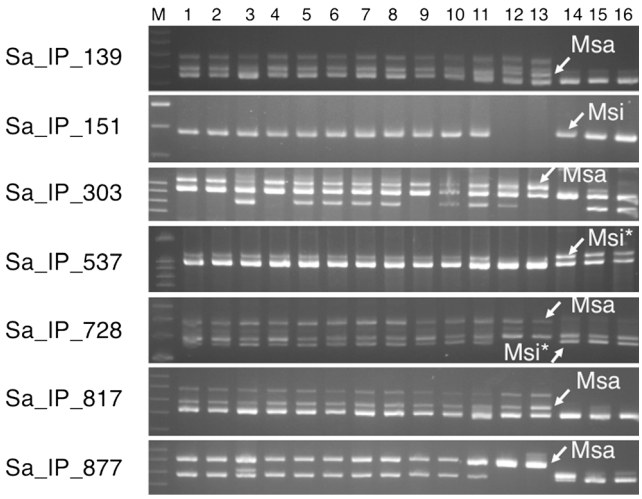

Fig. 4 Genotyping by the intron-flanking markers showing fragments specific to *Miscanthus sacchariflorus* (*Msa*) or *Miscanthus sinensis* (*Msi*) [28]. *Asterisks* indicate fragments detected in all *M. sinensis* genotypes and in some *M. sacchariflorus* genotypes in the study of Tamura et al. [28]. *M*, 100-bp ladder marker; *1–10*, randomly selected putative *Miscanthus* plants; *11*, *M.* ×*giganteus* 'Illinois'; *12*, *M. sacchariflorus* 'Uchinoura'; *13*, *M. sacchariflorus* 'Miyakonojyo'; *14–16*, *M. sinensis* genotypes collected in the Tashiro–Fumoto area

within each type. There were no differences between types A and C, or between types B1 and B2 (Table 2). There was only one difference (insertion–deletion in the *trnL-trnF* region) between the A + C and B groups (Table 2). All mutations identified in the putative hybrids were also present in *M. sacchariflorus* plants collected in Japan but not in *M. sinensis* plants in Tashiro–Fumoto area (Table 2). Mutations at the six loci detected in putative hybrids (Table 2) were not reported by Shimono et al. [32], who investigated chloroplast DNA variations in 636 *M. sinensis* individuals in Japan. The haplotype of types A+C was the same as that of *M. sacchariflorus* 'Morioka', whereas the haplotype of type B was same as those of *M. sacchariflorus* 'JM0126,' 'Hirosegawa,' and 'Kumagawa' (Table 2). Namely, two chloroplast haplotypes found in the putative hybrids were common to some *M. sacchariflorus* genotypes but not found in any *M. sinensis* genotypes.

Phenotypic Characterization of a Putative Hybrid *Miscanthus* Population in the Tashiro–Fumoto Area

Most florets of type B plants (B1 & B2) had awns (≥95 %) (Table 3). In contrast, only 25 % of florets of type C plants had awns (Table 3). Despite a wide variance, type A were intermediate with 36.3 % of florets with awns (Table 3).

Acetocarmine staining showed that type A plants had relatively high pollen viability, although there was variation among samples (Fig. 6a). In contrast, most pollen grains of type B1 plants were not viable (Fig. 6b). The difference between types A (73.2 %, Fig. 6c) and B1 (7.1 %, Fig. 6d) was significant ($P<0.001$, Wilcoxon's test).

To examine the possibility of expansion of the putative hybrid *Miscanthus* population by seed propagation, we investigated the seed fertility of putative hybrids and *M. sinensis* plants growing sympatrically. All panicles collected from putative hybrids contained filled seeds. The seed-set rate of type A plants was significantly lower than those of type B1 and *M. sinensis* (Table 4). Seedlings were raised from five of the nine samples of type A and all four samples of type B. The

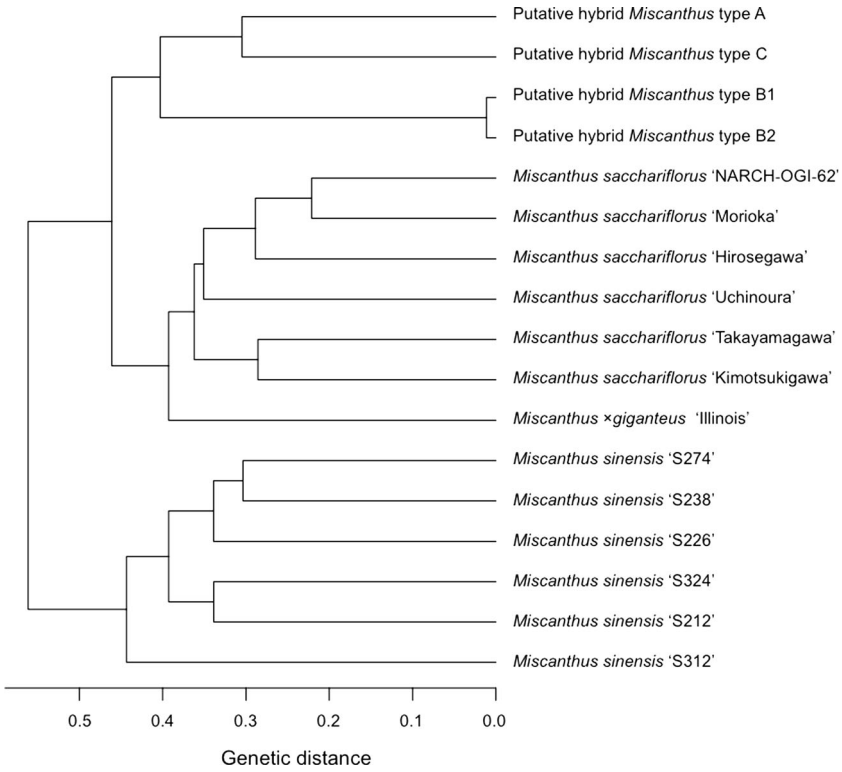

Fig. 5 Phylogenetic dendrogram of putative triploid hybrids between *M. sacchariflorus* and *M. sinensis* from Tashiro–Fumoto , and other *Miscanthus*. Phylogenetic analysis was based on polymorphisms detected by using 24 SSR markers

germination rate of the putative hybrid seeds (0.4 to 2.1 %) was marginally lower than that of *M. sinensis* seeds (Table 4).

The nuclear DNA content of 26 progeny obtained from eight parents of types A and B1 varied widely, ranging from 0.29 to 0.51 relative to *F. arundinacea*, which corresponded to 0.94 to 1.68 relative to diploid *M. sinensis* (Fig. S2). The mean nuclear DNA contents were not significantly different between progeny of types A (n=14) and B1 (n=12).

Discussion

The Hybrids Identified in the Tashiro–Fumoto Area are Triploid With a Tetraploid *M. Sacchariflorus* as Maternal Parent

Since the 1930s, several studies have reported naturally growing hybrids between sympatric *M. sacchariflorus* and *M. sinensis* in East Asia [4, 9, 15, 16]. However, these studies focused on individual plants, not populations. In this study, we found a large population of plants morphologically intermediate between *M sacchariflorus* and *M. sinensis*. The results of our cytological and genetic analyses indicate that the intermediate type of *Miscanthus* in the Tashiro–Fumoto area is a triploid hybrid between *M. sacchariflorus* and *M. sinensis*.

The genomic DNA contents of the parents are 4.5 pg per nucleus in *M. sacchariflorus* and 5.5 pg in *M. sinensis* [8]. On this basis, the DNA content of a triploid hybrid containing two *M. sacchariflorus* and one *M. sinensis* genomes is 1.32× that of diploid *M. sinensis*, and that of a triploid hybrid containing one *M. sacchariflorus* and two *M. sinensis* genomes is 1.41×. The mean DNA content of putative hybrids from Tashiro–Fumoto, 1.30, suggests that these plants are hybrids between tetraploid *M. sacchariflorus* and diploid *M. sinensis*, similar to *M. ×giganteus* [8, 9]. Similar to other triploid *Miscanthus* hybrids (*M. ×giganteus*) [18, 34], putative hybrids from Tashiro–Fumoto had *M. sacchariflorus*-specific haplotypes in the chloroplast genome. This suggests that maternal parents of the hybrids from Tashiro–Fumoto were tetraploid *M. sacchariflorus*.

Process of Emergence and Dispersal of This Triploid *Miscanthus* Hybrid Population

Why and how has this large (kilometer scale) hybrid population formed? In the Tashiro–Fumoto area, we found *M. sinensis* but no *M. sacchariflorus*. The native distribution of *M. sacchariflorus* is limited to the northern range of *M. sinensis*, which extends much further south to the tropics [1, 35]. The Global Biodiversity Information Facility (www.gbif.org) records the most southerly occurrence of *M. sacchariflorus* as 29.57°N in China, further south than the Tashiro–Fumoto area (31.2°N). However, as far as we investigated, the southern limit of *M. sacchariflorus* in Japan is ca. 9 km north of the Tashiro–Fumoto area (31.3°N, data not shown). The Tashiro–Fumoto area lies about 150–180 m

Table 2 Polymorphic sites in four chloroplast DNA regions of putative hybrid *Miscanthus*, *Miscanthus sinensis* from the Tashiro–Fumoto area, and *Miscanthus sacchariflorus* from southern Kyushu and other regions of Japan

Species	Accession	Location	Latitude	Longitude	*psbC-trnS*		*trnS-trnT*					*trnL-trnF*						
			(°N)	(°E)	442	1159	57	212	394	901	1002	221	339	344	619[b]	85	180	679
Putative *Miscanthus* hybrids in Tashiro–Fumoto [a]	Type A	Tashiro–Fumoto area, Kyushu	–	–	C	G[c]	C[c]	6 bp[d]	G	T	–[c]	G[c]	C	T	Indel_ type III[c]	–	G	A[c]
	Type B1	Tashiro–Fumoto area, Kyushu	–	–	C	G	C	6 bp	G	T	–	G	C	T	Indel_ type I[c]	–	G	A
	Type B2	Tashiro–Fumoto area, Kyushu	–	–	C	G	C	6 bp	G	T	–	G	C	T	Indel_ type I	–	G	A
	Type C	Tashiro–Fumoto area, Kyushu	–	–	C	G	C	6 bp	G	T	–	G	C	T	Indel_ type III	–	G	A
Miscanthus sinensis	S212	Tashiro–Fumoto area, Kyushu	31.2	130.9	C	A	A	–	G	T	17 bp[e]	T	C	A	Indel_ type VII	–	T	C
	S226	Tashiro–Fumoto area, Kyushu	31.2	130.8	C	A	A	–	G	T	17 bp	T	C	A	Indel_ type VII	–	T	C
	S238	Tashiro–Fumoto area, Kyushu	31.2	130.8	C	A	A	–	A	T	17 bp	T	A	A	Indel_ type VI	–	T	C
	S274	Tashiro–Fumoto area, Kyushu	31.2	130.9	C	A	A	–	G	T	17 bp	T	C	A	Indel_ type VII	–	T	C
	S312	Tashiro–Fumoto area, Kyushu	31.2	130.9	T	A	A	–	A	T	17 bp	T	A	A	Indel_ type VI	–	T	C
	S324	Tashiro–Fumoto area, Kyushu	31.2	130.9	C	A	A	–	G	T	17 bp	T	C	A	Indel_ type VII	–	T	C
Miscanthus sacchariflorus	Uchinoura	Kimotsuki-cho, Kyushu	31.3	131.1	C	G	C	6 bp	G	T	–	G	C	T	Indel_ type II	–	G	A
	Takayamagawa	Kimotsuki-cho, Kyushu	31.3	130.9	C	A	A	–	G	T	17 bp	T	C	A	Indel_ type VIII	5 bp[f]	G	C
	Kimotsukigawa	Kimotsuki-cho, Kyushu	31.4	131.0	C	G	C	6 bp	G	T	–	G	C	T	Indel_ type II	–	G	A
	JM0126	Miyakonojyo, Kyushu	31.5	131.0	C	G	C	6 bp	G	T	–	G	C	T	Indel_ type I	–	G	A
	Hirosegawa	Nichinan, Kyushu	31.6	131.4	C	G	C	6 bp	G	T	–	G	C	T	Indel_ type I	–	G	A
	Gotandagawa	Ichiki-Kushikino, Kyushu	31.7	130.3	T	A	A	–	A	T	17 bp	T	A	A	Indel_ type VI	–	G	C
	Sendaigawa	Satsuma-Sendai, Kyushu	31.8	130.3	C	A	C	6 bp	G	G	–	G	C	T	Indel_ type V	–	G	A
	Kumagawa	Sagara-mura, Kyushu	32.2	130.8	C	G	C	6 bp	G	T	–	G	C	T	Indel_ type I	–	G	A
	Morioka	Morioka, Tohoku	39.8	141.1	C	G	C	6 bp	G	T	–	G	C	T	Indel_ type III	–	G	A
	NARCH-OGI-62	Sapporo, Hokkaido	43.0	141.4	C	G	C	6 bp	G	T	–	G	C	T	Indel_ type IV	–	G	A

[a] Six samples each of types A and B1 were randomly selected from different points and sequenced. One sample each of types B2 and C was sequenced.

[b] Nucleotide sequence of each insertion-deletion is indicated in Figure S1.

[c] Mutations in putative hybrids not found by Shimono et al. [33] in *M. sinensis* collected in Japan.

[d] 6-bp insertion: GGGGAA

[e] 17-bp insertion: AGTAACACAAAAAATGG

[f] 5-bp insertion: TAACC

Table 3 Proportions of florets with awns in the putative hybrid *Miscanthus* population in Tashiro–Fumoto

		Florets with awn (%)	
Tested plants	No. plants	Mean	SD
Type A	15	36.3 [a]	26.6
Type B1	10	98.6 [b]	1.6
Type B2	2	95.0 [b]	3.9
Type C	2	25.0 [a]	8.6

Values followed by the different superscript letters are significantly different ($P<0.01$, Tukey's HSD test).

Table 4 Seed-set rate and germination rate of the putative hybrid *Miscanthus* population in Tashiro–Fumoto

		Seed-set rate (%)†		Germination rate (%)‡	
Tested plants	No. of plants	Mean	SD	Mean	SD
Type A	9	16.4 [a]	8.2	2.1	5.1
Type B1	4	39.3 [b]	11.3	0.4	0.4
Miscanthus sinensis	3	36.1 [b]	16.9	8.3	12.5

†Estimated by X-ray inspection.

‡Number of germinated seeds × 100 / (total number of tested seeds × seed-set rate).

Values followed by the different superscript letters are significantly different ($P<0.01$, Tukey's HSD test).

above sea level, and the temperature is a little cooler than in the surrounding lowlands. Therefore, we suppose that the parental *M. sacchariflorus* had grown in this area in the not-too-distant past before dying out owing to environmental changes such as warming, while the triploid hybrids survived owing to better adaptability introduced from *M. sinensis*. Clark et al. [36] reported that the genome of tetraploid *M. sacchariflorus* in Japan has introgressions from *M. sinensis* (on average 7 % of a whole genome), which are more frequent in southern Japan than in the northern region. They suggested that the ancestral *M. sinensis* introgression into *M. sacchariflorus* contributed to the adaptation to a warmer environment [36]. The existence of a large hybrid *Miscanthus* population at the southern limit of the range of *M. sacchariflorus* fits this hypothesis as an extreme example of the *M. sinensis* genome contributing to adaptation.

Following its establishment, how did the hybrid population expand to the landscape scale? On the one hand, we found that the genetic distances among types A, C and B were

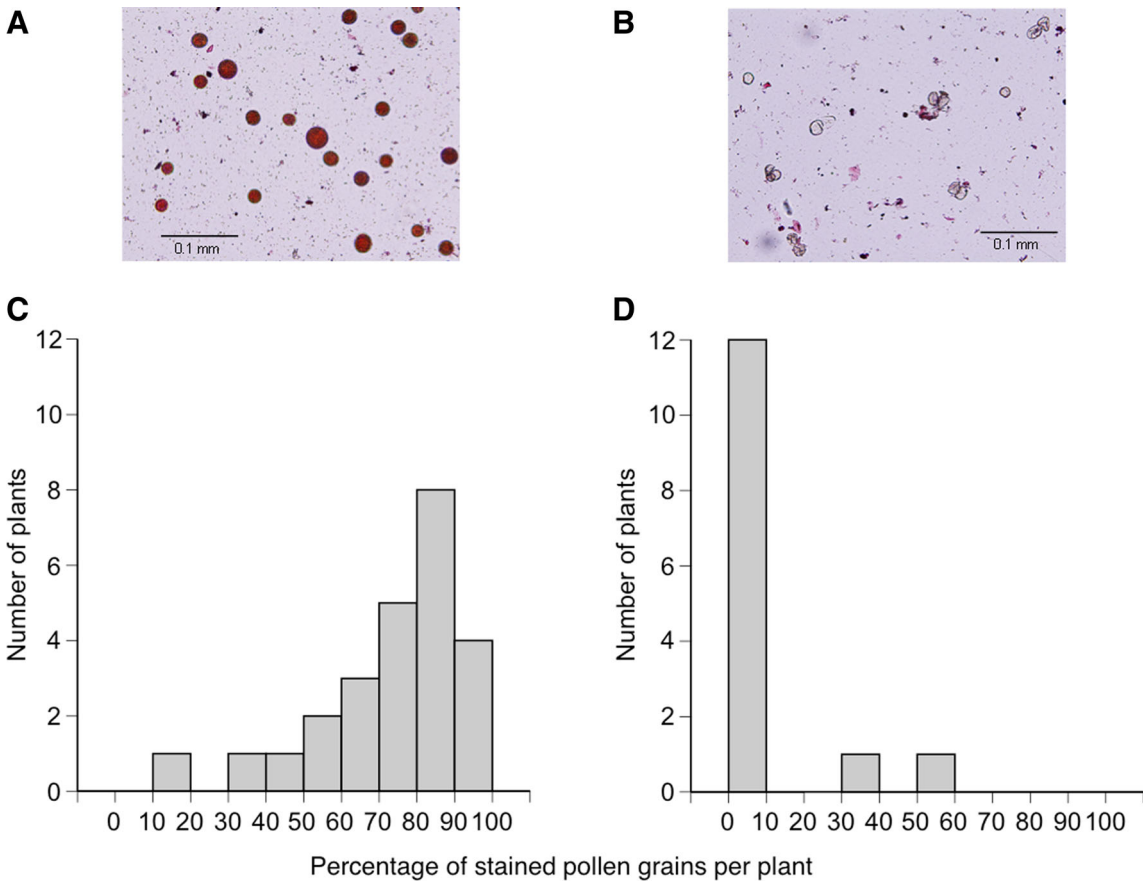

Fig. 6 Pollen viability of putative triploid hybrids between *M. sacchariflorus* and *M. sinensis* from Tashiro–Fumoto assessed by acetocarmine staining. **a** Type A. **b** Type B1. **c**, **d** Frequencies of stained pollen grains of (**c**) type A ($n=25$) and (**d**) type B (B1 + B2; $n=14$)

comparable to those among *M. sinensis* from Tashiro–Fumoto and among *M. sacchariflorus* in southern Kyushu. Mutations in chloroplast genomes were also found among the types of putative hybrids. These results demonstrate that the hybrids in the Tashiro–Fumoto area have a polyphyletic origin; namely, they originated from progeny of multiple pairs of *M. sacchariflorus* and *M. sinensis*. On the other hand, plants with the same genotype typically formed clusters. The simple genomic and geographic structures (Fig. 1c) imply that the population propagated clonally from a few (probably F_1) genotypes. In *M. sacchariflorus*, vegetative rhizomes are the main reproductive source for population recruitment [37–39]. Rhizome systems can be fragmented and dispersed by natural or human disturbance such as flooding or roadside maintenance [37, 39] and are recognized as a potential invasive risk in *M. ×giganteus*, especially in riparian ecosystems [40, 41]. According to a chronicle of the history of the area that includes Tashiro–Fumoto, the Fumoto River used to meander and often flooded following heavy rains, but 15–30 years ago it was channelized. The hybrid population area corresponds to the site of these works. Therefore, rhizomes of a few hybrid genotypes (mainly A and B1) might have been fragmented and spread in soil disturbed by floods, river improvement works, or both. Moreover, such habitat disturbances might have not only dispersed the propagules, but also led to the new conditions to which plants may become adapted following hybridization, as observed in other species [e.g., 23]. It is unlikely that the hybrid plants expanded through seed propagation, because they are triploid, and only four types were found by SSR marker analysis. Expansion through seed dispersal would result in a more heterogeneous genetic background and ploidy level, as confirmed in the plants raised from seeds of hybrid plants in this study.

Suggestions Regarding the Classification of *Miscanthus* Hybrids

The triploid hybrid *M. ×giganteus* has no awns, but the putative triploid hybrid *M. ×ogiformis* [11, 15, 16] has awns [15, 17]. On this basis, the Tashiro–Fumoto triploid hybrids should be classified as *M. ×ogiformis* rather than *M. ×giganteus*, although Linde-Laursen [11] insisted that *M. ×giganteus* should be taxonomically reclassified as *M. ×ogiformis*. However, the presence or absence of awns in the hybrids seems to be genetically variable, as seen in types A and B of the putative hybrids, and to be influenced by environment, as seen in the large variance among type A plants. Therefore, we think that the presence or absence of awns is not a reliable trait for classification.

Conclusion and Prospects

A landscape-scale population of triploid *Miscanthus* hybrids was discovered in the southern limit area of the range of *M. sacchariflorus*. We have proposed the hypothesis that under the environment where the growth of *M. sacchariflorus* was limited, hybridization between *M. sacchariflorus* and *M. sinensis* had an advantage due to its improved adaptability. Furthermore, genetic and phenotypic characterization suggest the polyphyletic origin and clonal propagation of this population. Further detailed studies, including genomic comparison with *Miscanthus* plants growing around this area and evaluation of the environmental adaptability of the hybrids, could test this hypothesis.

Natural *Miscanthus* hybrids adapted to different environments could be used as materials for breeding biomass crops. In sympatric areas where flowering times of *M. sacchariflorus* and *M. sinensis* overlap, such as in central to southern Japan, *Miscanthus* hybrid populations could occur; in fact, we have confirmed some other hybrid populations in Kyushu (unpublished data). As triploid hybrids can resemble *M. sacchariflorus* in natural habitats, previously collected *M. sacchariflorus* germplasms may include natural hybrids or plants with introgression from *M. sinensis*, which requires further inspections in the future.

Such natural hybrids, i.e., hybrids we found in a natural environment and that occurred spontaneously, set some seeds, some of which germinated. Although further testing is required to evaluate fertility accurately, these results show that the putative hybrids have partial paternal or maternal fertility. Type A plants in particular could be used as a pollen source to genetically improve hybrid *Miscanthus* as a biomass crop.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no potential conflict of interest.

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