

MISCANTHUS: INTER- AND INTRASPECIFIC GENOME SIZE VARIATION AMONG *M. ×GIGANTEUS*, *M. SINENSIS*, *M. SACCHARIFLORUS* ACCESSIONS

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Since *M. sinensis* Anderss., *M. sacchariflorus* (Maxim.) Hack. and *M. ×giganteus* J.M.Greef & Deuter ex Hodk. and Renvoize have considerably the highest potential for biomass production among *Miscanthus* Anderss. species, there is an urgent need to broaden the knowledge about cytological characteristics required for their improvement. In this study our objectives were to assess the genome size variation among eighteen *Miscanthus* accessions, as well as estimation of the monoploid genome size (2C and Cx) of the *M. sinensis* cultivars, which have not been analyzed yet. The characterization of three *Miscanthus* species was performed with the use of flow cytometry and analysis of the stomatal length. The triploid ($2n = 3x = 57$) *M. sinensis* 'Goliath' and *M. ×giganteus* clones possessed the highest 2C DNA content (8.34 pg and 7.43 pg, respectively). The intermediate 2C-values were found in the nuclei of the diploid ($2n = 2x = 38$) *M. sinensis* accessions (5.52–5.72 pg), whereas they were the lowest in the diploid ($2n = 2x = 38$) *M. sacchariflorus* ecotypes (4.58–4.59 pg). The presented study revealed interspecific variation of nuclear DNA content ($P < 0.01$) and therefore allowed for recognition of particular taxa, inter- and intraspecific hybrids and prediction of potential parental components. Moreover, intraspecific genome size variation ($P < 0.01$) was observed in *M. sinensis* cultivars at 3.62%. The values of the stomatal size obtained for the triploid *M. ×giganteus* 'Great Britain' (mean 30.70 μm) or 'Canada' (mean 29.67 μm) and diploid *M. sinensis* 'Graziella' (mean 29.96 μm) did not differ significantly, therefore this parameter is not recommended for ploidy estimation.

Keywords: Flow cytometry, intraspecific variation, *Miscanthus*, nuclear DNA content, stomatal size

INTRODUCTION

The rising interest in development and production of renewable energy sources is caused by depletion of fossil fuels reserves and concerns about atmospheric greenhouse gas concentrations. Perennial C4 grasses are a potential source of biomass and biofuels production due to their high productivity and root system sequestering carbon in the soil (McLaughlin and Walsh, 1998). Numerous studies have recently indicated that *Miscanthus* Anderss. species are promising candidates for low input bioenergy production in temperate regions (Dohleman and Long, 2009; Jones, 2011).

The genus *Miscanthus* (family: Poaceae, tribe: Andropogoneae, subtribe: Saccharinae) originates from tropical and subtropical environments and has been recognized as an ornamental plant introduced to European and American conditions (Hodkinson et al., 2002c; Quinn et al., 2010). Since *M. sinensis* Anderss., *M. sacchariflorus* (Maxim.) Hack. and *M. ×giganteus* J.M.Greef & Deuter ex Hodk. and Renvoize have considerably the highest potential for biomass production among *Miscanthus* species, there is an urgent need to broaden the knowledge about cytogenetic and molecular characteristics required for crop improvement, especially because of the fact that *M. sinensis* and *M. sacchariflorus*

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can hybridize in both natural and artificial environments (Adati and Shiotani, 1962; Clifton-Brown et al., 2008; Jeżowski, 2008; Nishiwaki et al., 2011). *M. ×giganteus* is an example of such a hybridization event, which occurred in natural conditions between the diploid *M. sinensis* ($2n = 2x = 38$) and allotetraploid *M. sacchariflorus* ($2n = 4x = 76$) from sympatric populations. The biomass productivity of *M. ×giganteus* proved to exceed its parental components (Linde-Laursen, 1993; Hernández et al., 2001; Hodgkinson et al., 2002a, 2002b; Nishiwaki et al., 2011; Heaton et al., 2010).

At present several new subspecies, varieties, horticultural cultivars of *M. sinensis*, *M. sacchariflorus* and *M. ×giganteus* have been described (Chae, 2012; Głowacka et al., 2015). According to Meyer and Tchida (1999), since 1980 over 50 *Miscanthus* selections have been introduced, mainly from the United States and Germany. Currently, in the USA nursery market more than 85 *M. sinensis* cultivars are available (Sacks et al., 2013).

For particular bioenergy breeding programs species of the *Miscanthus* genus need to be evaluated in terms of ploidy and genome size. The measurement of DNA content (genome size) is frequently used for better understanding of genome evolution (Huang et al., 2013). Nuclear DNA content provides data mainly for comparative analyses in a variety of taxonomic levels and groups, phylogenetic associations and for the designing of sequencing projects (Li et al., 2013). Since it becomes possible to measure the DNA content of a single nucleus, researchers have reported variations among different species (interspecific). However, the occurrence of genome size variation below the species level (intraspecific) is still controversial and not analyzed in a satisfactory way (Özkan et al., 2010). Although many reports of this phenomenon published till date have recently been disproved as erroneous, there are several examples of intraspecific DNA content variation based on properly conducted studies (Trávníček et al., 2013). Flow cytometric analyses have been used not only for defining the variation in different taxa (e.g. soybean, sugarcane, puffball) but also in natural and synthetic hybrids recognition, which usually show mid-parent values of the genome size (Ohri, 1998; Rayburn et al., 2004, 2005; Nishiwaki et al., 2011; Zhang et al., 2012; Trávníček et al., 2013). Rayburn et al. (2009) provided evidence for the existence of interspecific variation of nuclear DNA content and confirmed the union of a $2x$ *M. sacchariflorus* and a $1x$ *M. sinensis* gamete for the formation of *M. ×giganteus*. The genome size of *M. ×giganteus* was found to be 7.0 pg, whereas *M. sacchariflorus* and *M. sinensis* – 4.5 and 5.5 pg, respectively (Rayburn et al., 2009). In these *Miscanthus* species no intraspecific variation in the nuclear DNA content was found, but it is worth emphasizing that only three cultivars of *M. sinensis* and *M. sacchariflorus* were studied. In

recent studies on a larger number of *Miscanthus* accessions all of which included nuclear DNA content analysis, some differences among species could be observed (Chae, 2012; Głowacka et al., 2015). The present study is the first attempt to investigate whether intraspecific nuclear DNA content variation exists in *Miscanthus* cultivars.

The ploidy of *Miscanthus* spp. varies from diploid to hexaploid. *M. sacchariflorus* accessions native to China are mainly diploids, while accessions native to Japan are polyploids, mostly tetraploids (Moon et al., 2013; Sacks et al., 2013). *M. sinensis* is typically diploid, but natural and artificial polyploids are available. One of such examples is a triploid cultivar known as 'Goliath' (Clifton-Brown et al., 2008; Sacks et al., 2013). According to Purdy et al. (2013), it is an intraspecific hybrid of *M. sinensis*, which was selected by Ernst Pagels as a vigorous seedling from crossing of unknown parents. Głowacka et al. (2010) suggested that this hybrid came from the crossing of diploid and tetraploid *M. sinensis* but it has not been confirmed. It has been cultivated as a 'large-type' horticultural cultivar since the 1970s (Purdy et al., 2013). In the genotype screening trials, to investigate the productivity of *Miscanthus* genotypes, *M. sinensis* 'Goliath' indicated the same or even higher biomass yields and showed better survival rate after the first winter than *M. ×giganteus* (Jørgensen and Muhs, 2001). What is more, recent investigations have documented the variation in flowering time in *M. sinensis* taxa, which allows for further hybrid generation and adaptation of varieties to different climatic conditions (Jensen et al., 2011).

Our objectives were to use flow cytometry for DNA content estimation in the *Miscanthus* accessions and ensure the correct accession identification. Of particular interest was determination of whether inter- and intraspecific nuclear DNA variation existed in *Miscanthus* species. The results were used to assess the monoploid genome size for *Miscanthus* genotypes, which have not been analyzed yet. The potential of the nuclear DNA content estimation for determination of different hybridization events leading to the formation of triploid *M. sinensis* 'Goliath' or *M. ×giganteus* was verified. The measurement of the stomatal length was performed to determine whether it is a reliable method for ploidy estimation in *Miscanthus* accessions.

MATERIALS AND METHODS

PLANT MATERIAL

Eighteen accessions of the *Miscanthus* (Anderss.) species available in the field collection at the PBAI – NRI (Plant Breeding and Acclimatization Institute –

TABLE 1. Nuclear DNA content, ploidy, chromosome number and stomatal size in accessions of *Miscanthus* species.

Species	Clone /Cultivar/ Ecotype name	Ploidy	Chromosome number (2n)	Stomatal size (µm)	Nuclear DNA content					
					(pg mean ± SD)		(Mbp ^c)		2C/2n (pg)	
					2C	1Cx	2C	1Cx	2C	1Cx
<i>M. ×giganteus</i>	'Canada'	3x	57	29.67 ± 2.88 de ^a	7.48 ± 0.04 b ^b	2.49 ± 0.01 e ^c	7312	2437	0.13	0.39
<i>M. ×giganteus</i>	'Great Britain'	3x	57	30.70 ± 1.99 c	7.45 ± 0.04 b	2.48 ± 0.01 e	7286	2429	0.13	0.39
<i>M. ×giganteus</i>	'Floridulus'	3x	57	31.52 ± 2.48 b	7.41 ± 0.07 b	2.47 ± 0.02 e	7243	2414	0.13	0.39
<i>M. ×giganteus</i>	'Germany'	3x	57	31.62 ± 2.20 b	7.38 ± 0.07 b	2.46 ± 0.02 e	7220	2407	0.13	0.39
<i>M. sinensis</i>	'Goliath'	3x	57	33.62 ± 2.13 a	8.34 ± 0.07 a	2.78 ± 0.02 cd	8158	2719	0.15	0.44
<i>M. sinensis</i>	'Sirene'	2x	38	27.52 ± 4.85 fg	5.72 ± 0.04 c	2.86 ± 0.02 a	5590	2795	0.15	0.30
<i>M. sinensis</i>	'Graziella'	2x	38	29.96 ± 3.29 cd	5.72 ± 0.03 c	2.86 ± 0.02 a	5594	2797	0.15	0.30
<i>M. sinensis</i>	'Variegatus'	2x	38	24.04 ± 1.98 j	5.72 ± 0.03 c	2.86 ± 0.02 a	5594	2797	0.15	0.30
<i>M. sinensis</i>	'Flamingo'	2x	38	27.96 ± 2.33 g	5.71 ± 0.02 c	2.85 ± 0.01 a	5582	2791	0.15	0.30
<i>M. sinensis</i>	'Zebrinus'	2x	38	26.37 ± 2.98 h	5.68 ± 0.03 cd	2.84 ± 0.02 ab	5551	2776	0.15	0.30
<i>M. sinensis</i>	'Kleine Fontäne'	2x	38	28.98 ± 2.26 ef	5.61 ± 0.03 de	2.81 ± 0.02 bc	5490	2745	0.15	0.30
<i>M. sinensis</i>	'Kleine Silberspinne'	2x	38	24.06 ± 2.02 j	5.59 ± 0.04 e	2.79 ± 0.02 cd	5465	2733	0.15	0.29
<i>M. sinensis</i>	'Gracillimus'	2x	38	23.57 ± 2.67 j	5.58 ± 0.04 ef	2.79 ± 0.02 cd	5455	2728	0.15	0.29
<i>M. sinensis</i>	'Rotsilber'	2x	38	25.28 ± 2.22 i	5.58 ± 0.02 ef	2.79 ± 0.01 cd	5457	2729	0.15	0.29
<i>M. sinensis</i>	'Pünktchen'	2x	38	25.57 ± 2.77 hi	5.56 ± 0.04 ef	2.78 ± 0.02 cd	5440	2720	0.15	0.29
<i>M. sinensis</i>	'Malepartus'	2x	38	27.94 ± 2.25 g	5.52 ± 0.01 f	2.76 ± 0.01 d	5401	2701	0.15	0.29
<i>M. sacchariflorus</i>	ecotype I	2x	38	25.17 ± 2.29 i	4.59 ± 0.01 g	2.29 ± 0.01 f	4489	2245	0.12	0.24
<i>M. sacchariflorus</i>	ecotype II	2x	38	24.92 ± 2.64 i	4.58 ± 0.03 g	2.29 ± 0.01 f	4479	2240	0.12	0.24

^aMeans with the same letter are not significantly different at P < 0.05 (RIR Tukey's test).

^bMeans with the same letter are not significantly different at P < 0.01 (RIR Tukey's test).

^c1 pg = 978 Mbp (Doležel et al., 2003).

National Research Institute, Bydgoszcz, Poland) were used (Cichorz et al., 2014). The material included: 4 clones of *M. ×giganteus*; 12 ornamental cultivars of *M. sinensis*; 2 ecotypes of *M. sacchariflorus* (Table 1). The studies were carried out during the vegetative season of 2012.

NUCLEAR DNA CONTENT MEASUREMENT

Flow cytometric analysis was performed on young leaves of different *Miscanthus* genotypes growing in the field. Plant material (0.2–1 cm²) was chopped simultaneously with a fragment of a leaf of internal standard with a sharp razor blade in a plastic Petri dish containing 1 cm³ of nuclei-isolation buffer (0.1 M Tris, 2.5 mM MgCl₂·6H₂O, 85 mM NaCl, 0.1% v/v Triton X-100; pH 7.0), supplemented with PI (50 µg·cm⁻³) and ribonuclease A (50 µg·cm⁻³). *Pisum sativum* 'Set' (2C = 9.11 pg; Sliwiska et al., 2005) was used as an internal standard for all the sam-

ples except *M. sinensis* 'Goliath', where indirect internal standard, *M. sinensis* 'Malepartus' (2C = 5.52 pg; this work), was used. For each sample, 7,000–9,000 nuclei were analyzed using a Partec CyFlow SL Green (Münster, Germany) flow cytometer, equipped with an air-cooled argon-ion laser with green light emission at 532 nm. Histograms were analyzed using the FloMax (Partec GmbH, Münster, Germany) software. For each accession, 5 plants were analyzed. Nuclear DNA content was calculated using the linear relationship between the ratio of 2C peak positions of *Miscanthus/P. sativum* (or *M. sinensis* 'Goliath'/*M. sinensis* 'Malepartus') on the histogram of the fluorescence intensities. 2C nuclear DNA contents (pg) were transformed to megabase pairs of nucleotides using the following conversion: 1 pg = 978 Mbp (Doležel et al., 2003). The mean CV (coefficient of variation) value of internal standard was 2.62–4.53% and 3.47–4.55% for the samples which were regarded as acceptable (Doležel et al., 2007).

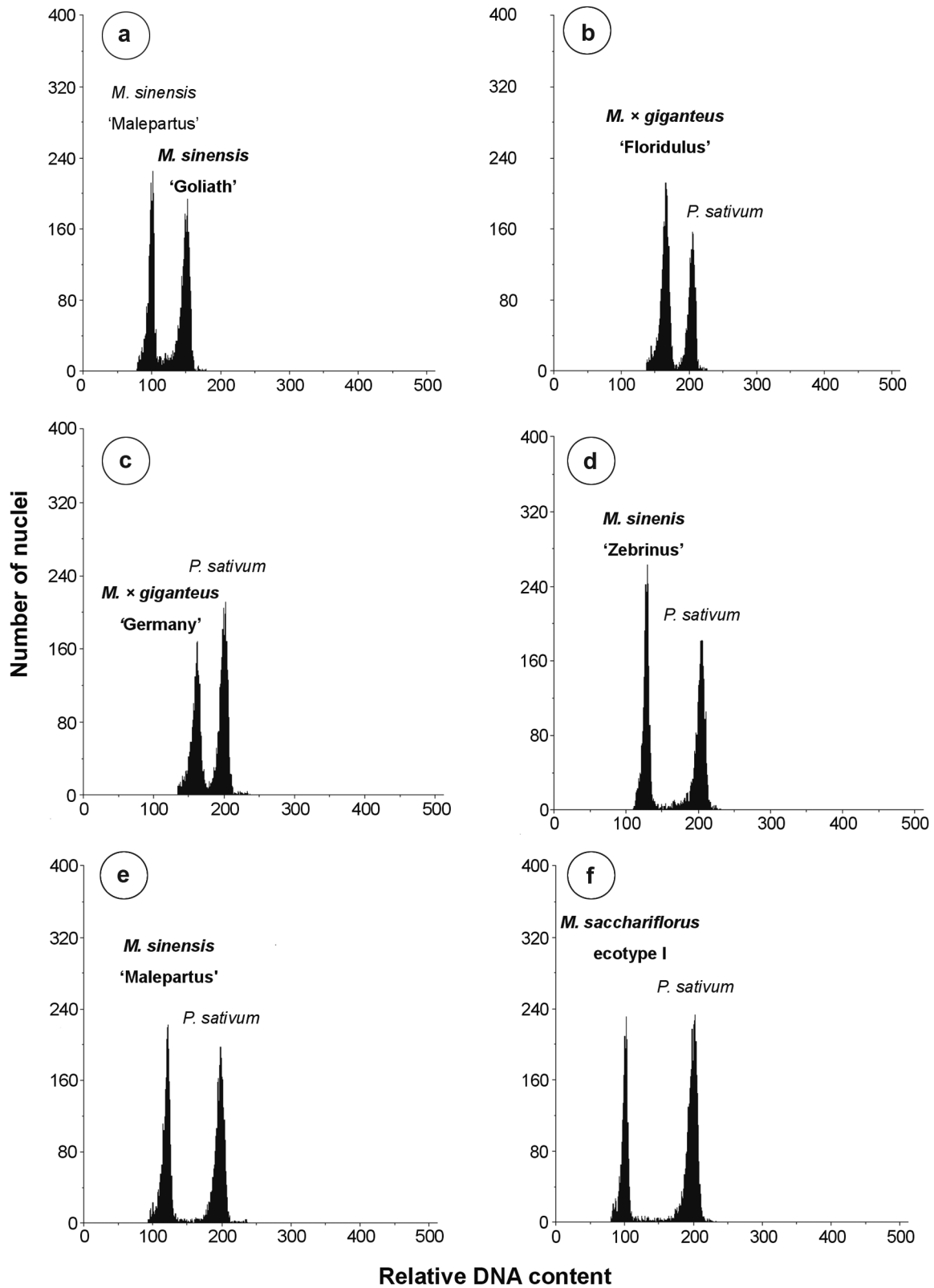


Fig. 1 Selected histograms of nuclear DNA content analysis of : (a) *M. sinensis* 'Goliath', (b) *M. × giganteus* 'Floridulus', (c) *M. × giganteus* 'Germany', (d) *M. sinensis* 'Zebrinus', (e) *M. sinensis* 'Malepartus', (f) *M. sacchariflorus* ecotype I.

STOMATAL SIZE MEASUREMENT

For comparison of stomatal size among plants with known ploidy, the epidermis from the abaxial leaf surface was peeled off with fine forceps, placed in a drop of Lugol's solution on a glass slide and covered with a microscope cover glass. According to Rayburn et al. (2009), stomatal size was measured in micrometers from the following parts of the leaf: tip, midsection and base. For each accession three leaves were analyzed and at least 167 measurements of stomatal size were made. Stomatal observations were performed using an optic microscope Jenamed 2, (Carl Zeiss Jena, Germany). The photographs were taken with a A101 cp camera (Basler Vision Technologies, Germany) and analyzed with Lucia – ScMeas ver. 4.51 software (Laboratory Imaging, Czech Republic).

CHROMOSOME COUNTS

Chromosome counts were made with slightly modified protocol of Chae (2012). Root and stem tips (5–10 mm long) were removed from the plants, placed in ice-chilled boxes and stored in 4°C for 24 h. After pre-treatment, the samples were fixed and stored in 3:1 (v/v) 100% ethanol/acetic acid at room temperature for four days. Root and stem tips were then stained with 2% orcein for 24 h. Meristems were macerated and analysed in 45% acetic acid. A cover slip was placed over the tissue and gently tapped with a dissecting needle to disperse the tissue. Chromosomes from three plants of each accession were counted. Observations were performed using an optic microscope Jenamed 2, (Carl Zeiss Jena, Germany).

STATISTICAL ANALYSIS

One-way ANOVA's and corresponding post-hoc RIR Tukey's and Tukey's HSD tests were used for each genotype to determine significant differences in nuclear DNA content ($P < 0.01$) and stomatal length ($P < 0.05$), respectively. Pearson's correlation coefficient was used to evaluate the correlation between the genome size, ploidy, stomatal size and chromosome number. The Statistica ver. 7.0 (StatSoft, Poland) software package was used for data management and statistical calculations.

RESULTS

For all the analyzed *Miscanthus* species the results of the 2C DNA content measurements enabled the authors to establish unambiguously the ploidy and taxonomic status of each accession (Table 1). The 2C DNA values for *Miscanthus* spp. ranged from 4.58 pg to 8.34 pg. The highest 2C DNA content was estimated in the nuclei of triploid ($2n = 3x = 57$) *M. sinensis* 'Goliath' (8.34 pg; Fig. 1a) and *M. ×gigan-*

teus clones (7.43 pg; Fig. 1b, 1c). The intermediate 2C-values were found in the nuclei of diploid ($2n = 2x = 38$) *M. sinensis* accessions (5.52–5.72 pg; Fig. 1d, 1e), whereas the lowest ones were observed in diploid ($2n = 2x = 38$) *M. sacchariflorus* ecotypes (4.58–4.59 pg; Fig. 1f). The triploids contained from 1.3- to 1.8-fold more 2C DNA than the diploids. The interspecific nuclear DNA content variation ($P < 0.01$) was observed for three analyzed species. Moreover, the intraspecific nuclear DNA content variation ($P < 0.01$) was found among the *M. sinensis* cultivars, whereas no intraspecific variation was observed for *M. ×giganteus* clones and *M. sacchariflorus* ecotypes. Six homogenous groups were revealed inside the *Miscanthus* genus (Table 1). The first group was represented only by triploid *M. sinensis* 'Goliath', which showed significant difference in 2C DNA content in comparison to *M. ×giganteus* clones, belonging to the second group. This confirmed a distinct hybrid nature of the above mentioned accessions. The third group was represented by *M. sinensis* cultivars: 'Sirene', 'Graziella', 'Variegatus', 'Flamingo'. The fourth group ('Zebrinus', 'Kleine Fontäne') showed intermediate values of the analyzed parameters between the third and fifth group ('Kleine Silberspinne', 'Gracilliums', 'Rotsilber', 'Pünktchen', 'Malepartus'). The sixth group consisted of *M. sacchariflorus* ecotypes. The highest monoploid genome size (1Cx) was observed in *M. sinensis* cultivars (the mean value of 2.81 pg), intermediate in *M. ×giganteus* clones (2.48 pg) and the smallest in *M. sacchariflorus* ecotypes (2.29 pg). The interspecific differences in 1Cx value among *Miscanthus* accessions were statistically significant ($P < 0.01$).

The stomatal length was measured for all the accessions and the analysis of variance indicated significant differences between these genotypes at $P < 0.05$ (Table 1, Fig. 2a–d). The mean stomatal size for: *M. ×giganteus* clones was 30.88 μm , *M. sacchariflorus* ecotypes 25.05 μm and for *M. sinensis* cultivars 27.10 μm . The highest value of stomatal length was observed in *M. sinensis* 'Goliath' at 33.62 μm (Fig. 2a) and the lowest in *M. sinensis* 'Gracillimus' at 23.57 μm (Fig. 2b).

A significant correlation between nuclear DNA content, ploidy, stomatal length and chromosome number was found (Table 2). The correlation between stomatal length and nuclear DNA content or ploidy was strong, whereas between nuclear DNA content and ploidy or chromosome number it was very strong.

DISCUSSION

Although *Miscanthus* spp. have significant potential as a bioenergy crop, still little is known about the cytogenetic features of this taxon and only few

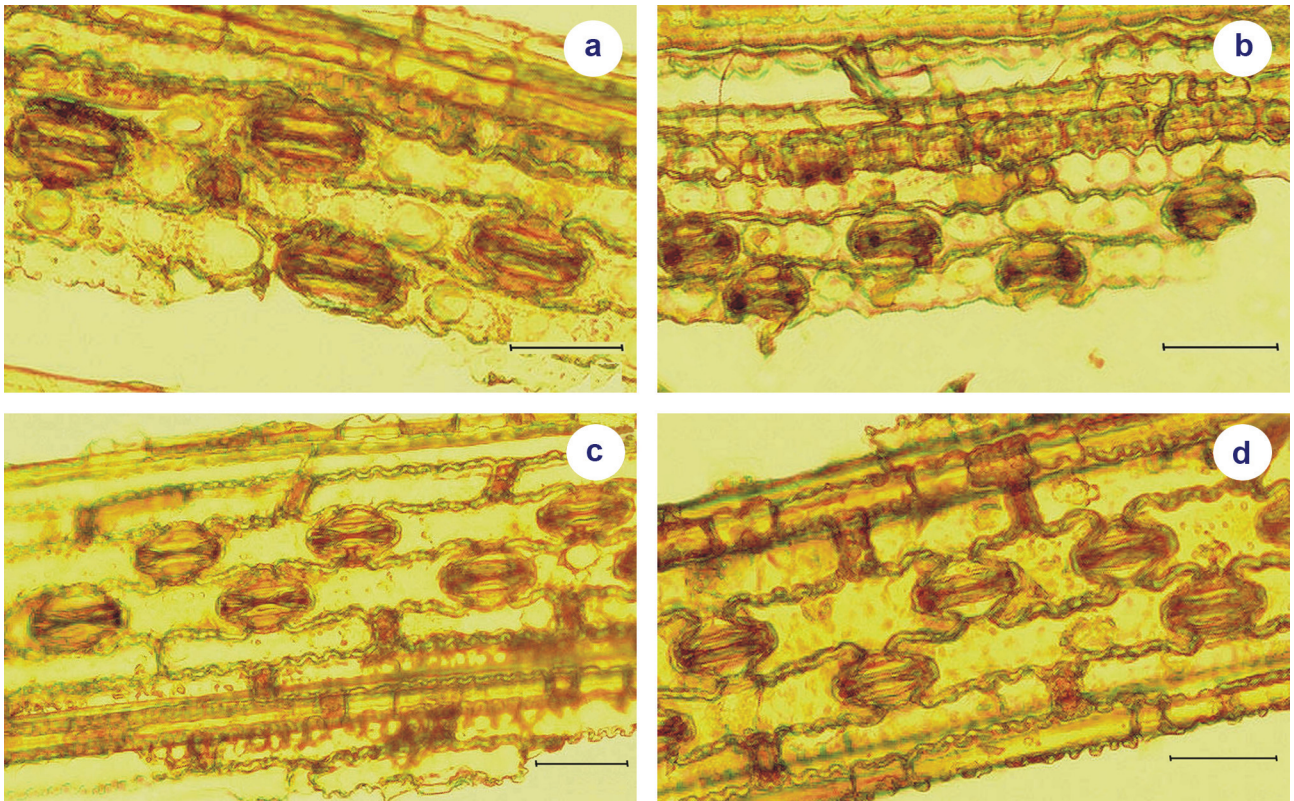


Fig. 2 Selected stomata photographs of: (a) *M. sinensis* 'Goliath' (triploid plant; mean stomatal length = 33.6 μm), (b) *M. sinensis* 'Gracillimus' (diploid plant; mean stomatal length = 23.6 μm), (c) *M. x giganteus* 'Germany' (triploid plant; mean stomatal length = 31.6 μm), (d) *M. x giganteus* 'Floridulus' (triploid plant; mean stomatal length = 31.5 μm). Bar = 30 μm .

reports are available. The nuclear DNA content estimation of this genus is advisable especially for conventional breeding programs and genomic studies or while searching for native environment for the valuable genotypes, especially natural hybrids (Rayburn et al., 2009; Nishiwaki et al., 2011). The taxonomic recognition of *Miscanthus* spp. mainly based on morphological features is dubious. The confusion sometimes arises during investigating, propagation and sampling germplasm collections (Hodkinson et al., 2002b). The errors in species identification may be caused by lack of taxonomic, molecular or cytological information and from sample mislabeling. Estimations of genome sizes of *Saccharum* germplasm helped to identify some mislabeled accessions (Zhang et al., 2012). Cytogenetic analysis performed by Chramiec-Głabik et al. (2012) revealed diploid nature of one *M. sinensis* accession, which, according to the breeder, was supposed to be a triploid. In the studies made by Chae (2012) the use of combined molecular markers and genome size information made it possible to relabel the misclassified *Miscanthus* accessions. In our study the flow cytometry analysis of nuclear DNA content indicated significant differences between particular

species, hence confirmed accession recognition. The 2C DNA contents received in this study were consistent with those previously published for *Miscanthus* (Rayburn et al., 2009; Swaminathan et al., 2010; Chramiec-Głabik et al., 2012; Chae, 2012; Moon et al., 2013; Li et al., 2013; Głowacka et al., 2015). However, some discrepancies were observed and slightly lower values appeared in the study published by Rayburn et al. (2009), which can be explained by the use of different nuclei-isolation buffer, internal standards and a flow cytometer (Doležel et al., 1998, 2005).

Of particular interest was determination of whether the inter- and intraspecific variation among *Miscanthus* spp. existed. Currently little is known about the prevalence of DNA content variation and the factors involved. The obtained results showed that differences in the genome size between *M. x giganteus*, *M. sinensis*, and *M. sacchariflorus* can be detected. Interestingly, they appear not only between accessions with divergent ploidy and chromosome number (*M. x giganteus* vs. *M. sacchariflorus*/*M. sinensis*) but also among species with the same values of the above cytogenetic characters (*M. sinensis* vs. *M. sacchariflorus* or *M. x giganteus*

TABLE 2. Coefficient of correlation between nuclear DNA content, ploidy, stomatal length and chromosome number of *Miscanthus* species.

Trait	Nuclear DNA content	Ploidy	Stomatal length
Ploidy	0.929 ^a	—	—
Stomatal length	0.543 ^a	0.609 ^a	—
Chromosome number	0.929 ^a	1.000 ^a	0.609 ^a

^aSignificantly different at $P < 0.05$ and $P < 0.01$

vs. *M. sinensis* 'Goliath'). The present experiment revealed that the nuclear DNA content of triploid *M. × giganteus* was approximately 32% and 62% higher than this of diploid *M. sinensis* and *M. sacchariflorus*, respectively. The difference appears to be due to the different chromosome number. However, at the diploid level *M. sacchariflorus* has ~0.5 pg/1Cx (~23 %) less DNA in comparison to *M. sinensis*. Similarly, 17–26% variation in 2C between the above mentioned species has been recently observed (Rayburn et al., 2009; Swaminathan et al., 2010; Nishiwaki et al., 2011; Chae, 2012; Li et al., 2013; Moon et al., 2013; Głowacka et al., 2015). The question arises: what are the main reasons which influenced 1.2-fold difference in nuclear DNA content between *M. sinensis* and *M. sacchariflorus* despite the same chromosome number? Many studies which compared features of these species have provided clear evidence on their distinct nature (Clifton-Brown and Lewandowski, 2000; Clifton-Brown et al., 2001; Zub et al., 2011, 2012). According to Hodkinson et al. (2002c), *M. sinensis* and *M. sacchariflorus* were grouped in separated phylogenetic subclades. As Li et al. (2013) suggested, on the basis of the relationship between *Miscanthus* and *Sorghum*, *M. sinensis* may be less evolutionarily advanced than *M. sacchariflorus*. Bennett (1987) stated that interspecific variation in DNA content plays an adaptive role and is correlated with geographical and environmental distribution of populations. Moreover, smaller genome size observed in *M. sacchariflorus* has its phenotypic consequences in early flowering. Diploid accessions of this species generally flower one or two months earlier in comparison with most *M. sinensis* genotypes, which complete the life cycle of those plants under a shorter growing season (Chae, 2012). From the biophysical point of view, the increase in the 2C DNA content causes not only the increase of nucleus and cell size, but also lengthening of the minimal time for cell division, which impacts the length of all stages of the cell cycle (Bennett and Leitch, 2005; Chae, 2012). A positive correlation between guard cell length and 2C DNA content was also indicated in

Miscanthus genotypes (Rayburn et al., 2009). As nuclear DNA content increases, the stomatal length also increases, which has been confirmed in this study. The *M. sinensis* 'Goliath' with the highest nuclear DNA content showed the widest stomatal size, whereas *M. sacchariflorus* ecotypes which possessed the smallest genome size, belonged to the group with the shortest stomatal size. In this research two ecotypes of *M. sacchariflorus* were compared with twelve *M. sinensis* ornamental cultivars of the higher phenotypic and genetic diversity. In native populations, *M. sacchariflorus* develops large genets mainly by spreading of the underground rhizomes rather than through sexual reproduction, which may probably affect lower diversity among these plants (Nishiwaki et al., 2011). If *M. sacchariflorus* produces seeds, its mass is smaller than in *M. sinensis*, which could be explained by smaller cell size and seed organs, such as cotyledons and hypocotyls (Li et al., 2013).

The presence of intraspecific variation has been widely studied in more than 80 genera and there are about 200 papers examining the evidence of its existence, yet it is still questioned (Šmarda and Bureš, 2010). Recent studies by Li et al. (2013) revealed that nuclear DNA contents were stable within natural populations of three *Miscanthus* species at the diploid level. The authors assumed that diverse growing environments did not cause any significant variability of DNA content. Rayburn et al. (2009), who analyzed the genome size of different cultivars of *M. sinensis* and *M. sacchariflorus*, did not detect intraspecific variation, either. Similar values of 2C DNA content for *M. sacchariflorus* cultivars ('Golf Course' – 4.40 pg; 'Robustus' – 4.43 pg) were received by Chae (2012) and Głowacka et al. (2015). These examples revealed that the difference in nuclear DNA content between the above mentioned cultivars was also very low (about 0.68%) and can be regarded as non-significant. However, if we take into consideration cultivars such as 'Bluemel' or 'Earthly Pursuits' – 4.29 pg and 'Hortico' – 4.47 pg, the difference rises to 4.2% (Głowacka et al., 2015). Furthermore, a comparison between two odd accessions belonging to *M. sinensis* var. *condensatus* 'Cosmopolitan' – 5.10 pg and 'Tripple Brook Farm' – 5.60 pg shows that the distinction reaches 9.8 % (Głowacka et al., 2015). In the research done by Chramiec-Głąbik et al. (2012), the difference between the genome size of *M. sinensis* MO7 (5.178 pg) and *M. sinensis* 'Gracillimus' (5.493 pg) was as high as 6.08 % and indicated statistical significance. These examples illustrated that intraspecific variation in nuclear DNA content could be observed among *M. sinensis* and *M. sacchariflorus* genotypes.

Our report is the first to confirm the intraspecific variation in nuclear DNA content among *M. sinensis* accessions, cultivated worldwide. The highest difference observed between cultivars with

extreme genome sizes ('Sirene' – 5.72 pg and 'Malepartus' – 5.52 pg) was estimated as 3.62%. Such variation (4%) between the two highest and lowest genome size lines belonging to the same species has been observed in soybean and considered to be small (Rayburn et al., 2004). The authors assumed that interspecies variation among soybean lines is due to the variation among the non-U.S. plant introductions (Rayburn et al., 2004). On the other hand, intraspecific variation between *M. sinensis* cultivars could be caused by different amounts of repetitive DNA, which seems to be primarily responsible for the large ranges of the genome size in plants (Bennett and Leitch, 2005). Especially the amplification and removal dynamics of retrotransposons are particularly important (SanMiguel and Bennetzen, 1998; Bennetzen et al., 2005; Zuccolo et al., 2007; Šmarda and Bureš, 2010). Further support for this hypothesis comes from the fact that much of repetitive DNA is composed of long terminal repeats (LTRs), which in grasses are clearly the most abundant. In recent studies, it was revealed that *M. sinensis* had genome-wide duplication, while in *M. ×giganteus* highly repetitive sequences represented the great majority of the genome (Swaminathan et al., 2010; Ma et al., 2012). That could probably explain the existence of intraspecific variation in nuclear DNA content among *M. sinensis* accessions used in our study, which was detectable by flow cytometric measurements, but did not affect the chromosome number.

Another aim of our study was to analyze if the DNA content makes it possible to distinguish odd hybrid nature, especially differences in parental components of two triploid accessions such as *M. ×giganteus* vs. *M. sinensis* 'Goliath' with the same chromosome number. In the previous study Rayburn et al. (2009) used nuclear DNA content estimation to determine the potential parental components of *M. ×giganteus*. The hypothetical result of the $2x M. sinensis \times 4x M. sacchariflorus$ crossing was estimated to have 7.2 pg, whereas *M. ×giganteus* accession revealed to have a nuclear DNA content of 7.0 pg. The authors assumed that the difference may be caused by the existence of intraspecific DNA variation among parental species. This means that different accessions with the nuclear DNA content that would precisely match the hypothetical value of *M. ×giganteus* could exist. In our study the predicted nuclear DNA content (7.40 pg) of the above mentioned species almost exactly matched the observed value (mean 7.43 pg); however, estimations made for *M. sinensis* 'Goliath' were not so straightforward. As was previously mentioned, 'Goliath' probably originated from a cross made between $2 \times M. sinensis \times 4 \times M. sinensis$. The estimated nuclear DNA content based on the mean

monoploid value (2.815 pg) of all *M. sinensis* cultivars (3×2.815 pg) would be 8.45 pg, while the observed one was 8.34 pg. This could be explained by the fact that in newly synthesized polyploids genome downsizing is a widespread phenomenon (Özkan et al., 2001; Leitch and Bennett, 2004). But if we considered the values obtained for 'Malepartus' and 'Rotsilber', $2 \times M. sinensis$ (5.52 pg) and $4 \times M. sinensis$ (2×5.58 pg), respectively, the calculated value would be exactly the same as the observed one (8.34 pg). Thus, maternal components of *M. sinensis* 'Goliath' could differ in nuclear DNA content, but are not indication of the maternal and paternal line de facto. The present results confirmed the usefulness of nuclear DNA content estimation as a helpful method of hybrid recognition between *Miscanthus* accessions with the same chromosome number but different parental components.

Since many breeding companies may not have the opportunity to analyze the ploidy in *Miscanthus* spp. by flow cytometry we have used a standard and easy method to perform measurements of the stomatal size. A high positive correlation between the ploidy and stomatal size suggests that the latter can be a suitable parameter for the preliminary identification of *Miscanthus* ploidy, which is in agreement with other results obtained by Glowacka et al. (2010). However, since the values of stomatal size obtained for triploid *M. ×giganteus* 'Great Britain' (mean 30.70 μm) or 'Canada' (mean 29.67 μm) and diploid *M. sinensis* 'Graziella' (mean 29.96 μm) did not differ significantly, using this parameter can cause confusion and mistakes. Therefore, flow cytometric measurement of *Miscanthus* ploidy is more reliable.

CONCLUSIONS

Since interspecific variation in the nuclear DNA content among *Miscanthus* species is rather high, recognition of particular taxa, inter- and intraspecific hybrids and prediction of the potential parental components is possible using flow cytometry. Although intraspecific variation in *M. sinensis* cultivars occurs, it is low and may be caused by the quantity rearrangements of retroelements content fixed during the breeding process. It needs, however, to be confirmed in further studies.

AUTHORS' CONTRIBUTIONS

Sandra Cichorz designed the experiment for flow cytometry and the stomatal size measurements, performed the statistical analysis, participated in interpretation of the results and wrote the first draft of the manuscript. Maria Goška performed the stom-

atal size measurement, chromosome counts and co-wrote the manuscript. Monika Rewers performed the flow cytometric analysis and participated in the evaluation of the result. The authors declare that there are no conflicts of interest.

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REFERENCES

- ADATI S, and SHIOTANI I. 1962. The cytotaxonomy of the genus *Miscanthus* and its phylogenic status. *Bulletin of the Faculty of Agriculture Mie University* 25: 1–24.
- BENNETT MD. 1987. Variation in genomic form in plants and its ecological implications. *New Phytologist* 106: 177–200.
- BENNETT MD, and LEITCH IJ. 2005. Plant genome size research: a field in focus. *Annals of Botany* 95: 1–6.
- BENNETZEN JL, MA J, and DEVOS KM. 2005. Mechanisms of recent genome size variation in flowering plants. *Annals of Botany* 95: 127–132.
- CHAE WB. 2012. Cytogenetics and genome structure in genus *Miscanthus*, a potential source of bioenergy feedstocks. Ph.D. dissertation, University of Illinois at Urbana-Champaign, Urbana, IL.
- CHRAMIEC-GLĄBIK A, GRABOWSKA-JOACHIMIĄK A, SŁIWINSKA E, LEGUTKO and J, KULA A. 2012. Cytogenetic analysis of *Miscanthus × giganteus* and its parent forms. *Caryologia* 65: 234–242.
- CLIFTON-BROWN JC, and LEWANDOWSKI I. 2000. Overwintering problems of newly established *Miscanthus* plantations can be overcome by identifying genotypes with improved rhizome cold tolerance. *New Phytologist* 148: 287–294.
- CLIFTON-BROWN JC, LEWANDOWSKI I, ANDERSSON B, BASCH G, CHRISTIAN DG, BONDERUP-KJELDSSEN J, JØRGENSEN U, MORTENSEN V, RICHE AB, SCHWARZ KU, TAYEBI K, and TEIXEIRA F. 2001. Performance of 15 *Miscanthus* genotypes at five sites in Europe. *Agronomy Journal* 93: 1013–1019.
- CLIFTON-BROWN JC, CHIANG YCH, and HODKINSON TR. 2008. *Miscanthus*: Genetic Resources and Breeding Potential to Enhance Bioenergy Production. In: Vermerris W [ed.], *Genetic Improvement of Bioenergy Crops*, 273–294. Springer, Gainesville, FL.
- CICHORZ S, GOŚKA M, and LITWINIEC A. 2014. *Miscanthus*: Genetic diversity and genotype identification using ISSR and RAPD markers. *Molecular Biotechnology* 56: 911–924.
- DOHLEMAN FG, and LONG SP. 2009. More productive than maize in the Midwest: how does *Miscanthus* do it? *Plant Physiology* 150: 2104–2115.
- DOLEŽEL J, GREILHUBER J, LUCRETTI S, MEISTER A, LYSÁK MA, NARDI L, and OBERMAYER R. 1998. Plant genome size estimation by flow cytometry: inter-laboratory comparison. *Annals of Botany* 82: 17–26.
- DOLEŽEL J, BARTOŠ J, VOGLMAYR H, and GREILHUBER J. 2003. Nuclear DNA content and genome size of trout and human. *Cytometry Part A* 51: 127–128.
- DOLEŽEL J and BARTOŠ J. 2005. Plant DNA flow cytometry and estimation of nuclear genome size. *Annals of Botany* 95: 99–110.
- DOLEŽEL J, GREILHUBER J, and SUDA J. 2007. Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols* 2: 2233–2244.
- GŁOWACKA K, JEŻOWSKI S, and KACZMAREK Z. 2010. *In vitro* induction of polyploidy by colchicine treatment of shoots and preliminary characterization of induced polyploids in two *Miscanthus* species. *Industrial Crops and Products* 32: 88–96.
- GŁOWACKA K, CLARK LV, ADHIKARI S, PENG J, STEWART JR, NISHIWAKI A, YAMADA T, JØRGENSEN U, HODKINSON TR, GIFFORD J, JUVIK JA, and SACKS EJ. 2015. Genetic variation in *Miscanthus × giganteus* and the importance of estimating genetic distance thresholds for differentiating clones. *Global Change Biology Bioenergy* 7: 386–404.
- HEATON EA, DOHLEMAN FG, MIGUEZ AF, JUVIK JA, LOZOVAYA V, WIDHOLM J, ZABOTINA OA, MCISAAC GF, and MARK B. 2010. *Miscanthus*: a promising biomass crop. *Advances in Botanical Research* 56: 75–137.
- HERNÁNDEZ P, DORADO G, LAURIE DA, MARTÍN A, and SNAPE JW. 2001. Microsatellites and RFLP probes from maize are efficient sources of molecular markers for the biomass energy crop *Miscanthus*. *Theoretical and Applied Genetics* 102: 616–622.
- HODKINSON TR, CHASE MW, TAKAHASHI C, LEITCH IJ, BENNETT MD, and RENVOIZE SA. 2002a. The use of DNA sequencing (ITS and *trnL-F*), AFLP, and fluorescent *in situ* hybridization to study allopolyploid *Miscanthus* (Poaceae). *American Journal of Botany* 89: 279–286.
- HODKINSON TR, CHASE MW, and RENVOIZE SA. 2002b. Characterization of a genetic resource collection for *Miscanthus* (Saccharinae, Andropogoneae, Poaceae) using AFLP and ISSR PCR. *Annals of Botany* 89: 627–636.
- HODKINSON TR, CHASE MW, LLEDO MD, SALAMIN N, and RENVOIZE SA. 2002c. Phylogenetics of *Miscanthus*, Saccharum and related genera (Saccharinae, Andropogoneae, Poaceae) based on DNA sequences from ITS nuclear ribosomal DNA and plastid *trnL* intron and *trnL-F* intergenic spacers. *Journal of Plant Research* 115: 381–392.
- HUANG H, TONG Y, ZHANG QJ, and GAO LZ. 2013. Genome size variation among and within *Camellia* species by using flow cytometric analysis. *PLoS ONE* 8(5): e64981. doi:10.1371/journal.pone.0064981.
- JENSEN E, FARRAR K, THOMAS-JONES S, HASTINGS A, DONNISON I, and CLIFTON-BROWN J. 2011. Characterization of flowering time diversity in *Miscanthus* species. *Global Change Biology Bioenergy* 3: 387–400.
- JEŻOWSKI S. 2008. Yield traits of six clones of *Miscanthus* in the first 3 years following planting in Poland. *Industrial Crops and Products* 27: 65–68.

- JONES MB. 2011. C4 Species as Energy Crops. In: Raghavendra AS, Sage RF [ed.], *C4 Photosynthesis and related CO₂ concentrating mechanisms*, 379–397. Springer Netherlands, Dordrecht, Netherlands.
- JØRGENSEN U, and MUHS HJ. 2001. *Miscanthus* breeding and improvement. In: Jones MB, Walsh M [ed.], *Miscanthus for energy and fiber*, 68–86. James and James, London, United Kingdom.
- LEITCH IJ, and BENNETT MD. 2004. Genome downsizing in polyploid plants. *Biological Journal of the Linnean Society* 82: 651–663.
- LI X, HU D, LUO M, ZHU M, LI X, LUO F, LI J, and YAN J. 2013. Nuclear DNA content variation of three *Miscanthus* species in China. *Genes & Genomics* 35: 13–20.
- LINDE-LAURSEN I. 1993. Cytogenetic analysis of *Miscanthus 'Giganteus'*, an interspecific hybrid. *Hereditas* 119: 297–300.
- MA XF, JENSEN E, ALEXANDROV N, TROUKHAN M, ZHANG L, THOMAS-JONES S, FARRAR K, CLIFTON-BROWN J, DONNISON I, SWALLER T, and FLAVELL R. 2012. High resolution genetic mapping by genome sequencing reveals genome duplication and tetraploid genetic structure of the diploid *Miscanthus sinensis*. *PLoS ONE* 7(3): e33821. doi:10.1371/journal.pone.0033821.
- McLAUGHLIN SB, and WALSH ME. 1998. Evaluating environmental consequences of producing herbaceous crops for bioenergy. *Biomass and Bioenergy* 14: 317–324.
- MEYER MH, and TCHIDA CL. 1999. *Miscanthus Anderss.* produces viable seed in four USDA hardiness zones. *Journal of Environmental Horticulture* 17: 137–140.
- MOON YH, CHA YL, CHOI YH, YOON YM, KOO BC, AHN JW, AN GH, KIM JK, and PARK KG. 2013. Diversity in ploidy levels and nuclear DNA amounts in Korean *Miscanthus* species. *Euphytica* 193: 317–326.
- NISHIWAKI A, MIZUGUTI A, KUWABARA S, TOMA Y, ISHIGAKI G, MIYASHITA T, YAMADA T, MATUURA H, YAMAGUCHI S, RAYBURN AL, AKASHI R, and STEWART JR. 2011. Discovery of natural *Miscanthus* (Poaceae) triploid plants in sympatric populations of *Miscanthus sacchariflorus* and *Miscanthus sinensis* in southern Japan. *American Journal of Botany* 98: 154–159.
- OHRI D. 1998. Genome size variation and plant systematics. *Annals of Botany* 82: 75–83.
- ÖZKAN H, LEVY AA, and FELDMAN M. 2001. Allopolyploidy-induced rapid genome evolution in the wheat (*Aegilops-Triticum*) group. *Plant Cell* 13: 1735–1747.
- ÖZKAN H, TUNA M, KILIAN B, MORI N, and OHTA S. 2010. Genome size variation in diploid and tetraploid wild wheats. *AoB Plants* doi:10.1093/aobpla/plq015.
- PURDY SJ, MADDISON AL, JONES LE, WEBSTER RJ, ANDRALOJC J, DONNISON I, and CLIFTON-BROWN J. 2013. Characterization of chilling-shock responses in four genotypes of *Miscanthus* reveals the superior tolerance of *M. ×giganteus* compared with *M. sinensis* and *M. sacchariflorus*. *Annals of Botany* 111: 999–1013.
- QUINN LD, ALLEN DJ, and STEWART JR. 2010. Invasiveness potential of *Miscanthus sinensis*: implications for bioenergy production in the United States. *Global Change Biology Bioenergy* 2: 310–320.
- RAYBURN AL, BIRADAR DP, NELSON RL, MCCLOSKEY R, and YEATER KM. 2004. Documenting intraspecific genome size variation in soybean. *Crop Science* 44: 261–264.
- RAYBURN AL, MCCLOSKEY R, TATUM TC, BOLLERO GA, JESCHKE MR, and TRANEL PJ. 2005. Genome size analysis of weedy *Amaranthus* species. *Crop Science* 45: 2557–2562.
- RAYBURN AL, CRAWFORD J, RAYBURN CM, and JUVIK JA. 2009. Genome size of three *Miscanthus* species. *Plant Molecular Biology Reporter* 27: 184–188.
- SACKS EJ, JUVIK JA, LIN Q, STEWART JR, and YAMADA T. 2013. The gene pool of *Miscanthus* species and its improvement. In: Paterson AH [ed.], *Genomics of the Saccharinae, Plant Genetics and Genomics: Crops and Models*, 73–100. Springer Science + Business Media, New York, NY.
- SANMIGUEL P, and BENNETZEN JL. 1998. Evidence that a recent increase in maize genome size was caused by the massive amplification of intergene retrotransposons. *Annals of Botany* 82: 37–44.
- SLIWINSKA E, ZIELINSKA E, and JEDRZEJCZYK I. 2005. Are seeds suitable for flow cytometric estimation of plant genome size? *Cytometry Part A* 64: 72–79.
- SWAMINATHAN K, ALABADY MS, VARALA K, DE PAOLI E, HO I, ROKHSAR DS, ARUMUGANATHAN AK, MING R, GREEN PJ, MEYERS BC, MOOSE SP, and HUDSON ME. 2010. Genomic and small RNA sequencing of *Miscanthus ×giganteus* shows the utility of sorghum as a reference genome sequence for *Andropogoneae* grasses. *Genome Biology* 11: R12. doi: 10.1186/gb-2010-11-2-r12.
- ŠMARDÁ P, and BUREŠ P. 2010. Understanding intraspecific genome size variation. *Preslia* 82: 41–61.
- TRÁVNÍČEK P, KIRSCHNER J, CHUDÁČKOVÁ H, ROOKS F, and ŠTÍPÁNEK J. 2013. Substantial genome size variation in *Taraxacum stenocephalum* (Asteraceae, Lactuceae). *Folia Geobotanica* 48: 271–284.
- ZHANG J, NAGAI C, YU Q, PAN YB, AYALA-SILVA T, SCHNELL RJ, COMSTOCK JC, ARUMUGANATHAN AK, and MING R. 2012. Genome size variation in three *Saccharum* species. *Euphytica* 185: 511–519.
- ZUB HW, ARNOULT A, and BRANCOURT-HULMEL M. 2011. Key traits for biomass production identified in different *Miscanthus* species at two harvest dates. *Biomass and Bioenergy* 35: 637–651.
- ZUB HW, RAMBAUD C, BÉTHENCOURT L, and BRANCOURT-HULMEL M. 2012. Late emergence and rapid growth maximize the plant development of *Miscanthus* clones. *BioEnergy Research* 5: 841–854.
- ZUCCOLO A, SEBASTIAN A, TALAG J, YU Y, KIM H, COLLURA K, KUDRNA D, and WING RA. 2007. Transposable element distribution, abundance and role in genome size variation in the genus *Oryza*. *BMC Evolutionary Biology* 7: 152 doi:10.1186/1471-2148-7-152.