Short communication

Comparison of relative DNA content estimated using DAPI and PI-FCM in Miscanthus sinensis, Miscanthus sacchariflorus, and their hybrids

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ABSTRACT Three triploid Miscanthus hybrids were found in Kushima, Japan, by a first screening of M. sinensis and M. sacchariflorus plants by using 4’,6-diamidino-2-phenylindole, dilactate dye flow cytometry (DAPI-FCM). The ploidy levels of the three triploid hybrids were confirmed by a second screening using propidium iodide dye flow cytometry (PI-FCM). It was possible that we might have missed a few triploid hybrids in the first screening because of a weak correlation of relative estimated DNA contents between PI-FCM and DAPI-FCM. Therefore, to get the conclusive evidence of three accessions newly discovered being triploid among some Miscanthus spp., we conducted a comparison of the relative DNA contents estimated with AT-selective DAPI dye and intercalary PI dye in diploid (2x) M. sinensis and tetraploid (4x) M. sacchariflorus and their triploid (3x) hybrids. A strong linear correlation was observed between both FCM analyses. The slope of the regression line was 1.15. This result shows the high reliability of the findings of our previous study on Miscanthus accessions.

Keywords: correlation, flow cytometry, fluorescence ratio, ploidy level, regression analysis

Introduction

Three triploid putative hybrids were found in sympatric populations of diploid (2x) Miscanthus sinensis and tetraploid (4x) M. sacchariflorus in Kushima, Japan (Nishiwaki et al. 2011). This discovery could alleviate the risks associated with the triploid (3x) hybrid M. ×giganteus Greef & Deuter ex Hodkinson and Renvoize (Clifton-Brown et al. 2001). These putative hybrids were confirmed as true hybrids by using morphological analysis and sequencing of the ribosomal DNA internal transcribed spacer (ITS) region (Dwiyanti et al. 2012). In our previous study, we screened for Miscanthus accessions collected from sympatric populations of the two Miscanthus species by using 4’,6-diamidino-2-phenylindole, dilactate dye flow cytometry (DAPI-FCM), and we detected three putative 3x hybrids (Nishiwaki et al. 2011). Then, we attempted to measure relative DNA content for only three putative 3x hybrids by using propidium iodide dye flow cytometry (PI-FCM) as second screening.

The choice of fluorochromes is primarily determined by the excitation source available. PI is excited by visible light with an absorbancy maximum at 490 nm, while DAPI is excited by UV light at 350 nm (Johnston et al. 1999). Many researchers used DAPI-FCM because equipments for PI-FCM were limited. But, Johnston et al. (1999) said that any initial genome size report based upon DAPI should be verified using a base ratio independent stain. Dolezel et al. (1992) reported that the use of dyes showing a base preference would lead to large errors in DNA content estimation. The two dyes have quite different stain reactions; fluorescence intensity intercalates between base pairs of double-stranded DNA and RNA with little or no base specificity (Properi et al. 1991). Compare to DAPI-based FCM, DNA content estimated by PI-based FCM produces very consistent results.
compare with DAPI-based one (Godelle et al. 1993). The relative value of DAPI is higher than expected in the case of the ratio of AT genomes is rich.

Although similar AT/GC ratios within a plant family result from the general similarity of DNA sequences within a family, different plant species vary with respect to the ratios of nucleotide base pairs of genomic DNA (Barow and Meister 2002). Johnston et al. (1999) recommended PI as a fluorochrome for the determination of DNA content using flow cytometry, and DAPI should be used only if the estimated DNA value is corroborated by using other stains that have no bias for AT- or GC-rich sequences in genomes.

Smarda and Bures (2006) confirmed the relationship between DAPI and PI staining in Festuca plants on the basis of measurements made in different seasons. DAPI could be used for estimating the absolute DNA content in Festuca. Correspondence and correlation measurements require accuracy and reproducibility of measurement for both DAPI and PI dyes. Dolezel and Bartus (2005) reported that correlation between both DAPI and PI flow dyes is required for all accessions to verify that precise results are obtained using FCM. The relationship between DAPI and PI staining and differences observed in the studied plants were confirmed on the basis of measurements in the same accessions.

However, in our previous study (Nishiwaki et al. 2011) we did not conduct simultaneous measurements of relative DNA content with comparisons between DAPI-FCM and PI-FCM for Miscanthus accessions. Thus, it is possible that we missed 3x hybrids in the first screening, if there is a weak correlation of the relative DNA contents between PI-FCM and DAPI-FCM. Therefore, we conducted a comparison of the relative DNA content estimated with AT-selective DAPI and intercalary PI in 2x M. sinensis and 4x M. sacchariflorus and their 3x hybrids.

**Materials and Methods**

**Plant Materials**

A set of plants was chosen, as previously reported by Nishiwaki et al. (2011), and it included three putative hybrids and M. ×giganteus. In addition, M. sinensis and M. sacchariflorus collected as mature plants from various localities in Japan (Tomakomai, Gifu, Tsukuba, Miyazaki and Kushima) were also used as samples in this study. These plants were previously assessed for relative DNA content by using DAPI-FCM. Then, we attempted to measure relative DNA content by using PI-FCM in 2012. All plants used in this experiment have been growing since 2009 at the Field Science Center of University of Miyazaki.

**Relative DNA content**

The relative DNA content of Miscanthus accessions from five populations was estimated. Flow cytometry with PI was used to confirm the relative DNA content estimated using DAPI-FCM for Miscanthus accessions (Nishiwaki et al. 2011). We did not conduct PI-FCM for Miscanthus accessions, with the exception of 3x hybrids (M. ×giganteus, Ogi63, Ogi79, and Ogi80).

Approximately 0.5 cm² of young leaf tissue from sorghum (Sorghum bicolor ‘Tx623’) (2C = 1.67 pg) was used as the internal standard, and 1.0 cm² of each of the Miscanthus accessions from five populations were placed in 1 mL of extraction buffer (50 mol L⁻¹ Tris-HCl, 0.5% (w/v) polyvinylpyrrolidone, 0.01% (v/v) Triton-X, 0.63% (w/v) sodium sulfite; pH 7.5) for 5 min and co-chopped with a razor blade. The mixture was then filtered through a 50-µm nylon mesh. After removal of the supernatant, the extraction buffer was supplemented with 100 µg mL⁻¹ of RNase. The suspension was then incubated at room temperature. Fifty µL of 0.1% PI solution was added to the suspension and incubated for 5 min at room temperature to stain the nuclei.

Fluorescence intensities of the samples were analyzed using a Cell Lab Quanta ™ SC MPL (Beckman Coulter, Tokyo Japan) equipped with a 488-nm argon laser. Relative DNA content was estimated by comparing the fluorescence intensities of the samples derived from the accessions to that of S. bicolor ‘Tx623’. Genome sizes of the accessions were also estimated by comparing the fluorescence intensities of the samples derived from the accessions to that of S. bicolor ‘Tx623’ (2C = 1.67 pg).

**Regression analysis**

The mean values of relative DNA content of Miscanthus accessions from five populations were calculated by three to six measurements of accessions. Mean values of relative DNA content of the 3x hybrids were calculated by three measurements per accession. Linear regression analysis between DAPI-FCM and PI-FCM data was performed using the regression analysis tool of Microsoft Excel 2012. The regression was evaluated with the coefficient of determination ($R^2$).
Results

Comparison between the relative DNA contents obtained using DAPI-FCM and PI-FCM for the Miscanthus accessions from five populations in Japan (Tomakomai, Tsukuba, Gifu, Miyazaki, and Kushima) is shown in Table 1.

Although the orders of relative DNA content in the 3x plants were slightly different in DAPI-FCM (Ogi79 < Ogi80 < M. × giganteus < Ogi63) and PI-FCM (Ogi79 < Ogi80 < Ogi63 < M. × giganteus), the orders of each value were very similar. The mean values of the 3x hybrids were very similar among accessions.

The orders of relative DNA content for M. sacchariflorus were almost the same between DAPI-FCM (Gifu < Tsukuba < Tomakomai < Miyazaki < Kushima) and PI-FCM (Gifu < Tsukuba = Tomakomai < Miyazaki < Kushima). The mean values of nuclear DNA content for M. sacchariflorus accessions were relatively large, varying from 8.08 pg/2C in Gifu to 8.90 pg/2C in Kushima.

The orders of relative DNA content for M. sinensis accessions were also very similar between DAPI-FCM and PI-FCM. Mean values of DNA content for accessions in M. sinensis were relatively small, varying from 5.06 pg/2C in Miyazaki to 5.29 pg/2C in Kushima. The mean values of relative DNA content for the Miscanthus accessions from five populations and 3x hybrids were slightly higher for PI-FCM than for DAPI-FCM in each accession. PI/DAPI ratios of many accessions were approximately 1.1.

A strong linear correlation appeared between DAPI- and PI-FCM (Figure 1). The value of the coefficient of determination of linear regression was $R^2 = 0.99152$, meaning that the AT contents of Miscanthus accessions were not significantly different from one another. The slope of the least-square regression line was 1.15. This result shows the slightly lower AT/GC ratio of Miscanthus accessions than of the internal standard S. bicolor ‘Tx623’.

![Figure 1](image)

**Table 1** Comparison of relative DNA content estimated using DAPI-FCM and PI-FCM; DNA content using internal standard (Sorghum bicolor ‘Tx623’; 2C = 1.67 pg); and PI/DAPI ratio of Miscanthus accessions.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Accessions</th>
<th>No. of measurements (No. of plants)</th>
<th>DAPI: Relative DNA content</th>
<th>S.D. †</th>
<th>Pl: Relative DNA content</th>
<th>S.D. †</th>
<th>DNA content (pg/2C, Using internal standard S. bicolor)</th>
<th>Ploidy level</th>
<th>PI/DAPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Illinois</td>
<td>M. × giganteus</td>
<td>3 (1)</td>
<td>3.80</td>
<td>0.03</td>
<td>4.18</td>
<td>0.10</td>
<td>6.98</td>
<td>3x</td>
<td>1.10</td>
</tr>
<tr>
<td>Kushima</td>
<td>Ogi63</td>
<td>3 (1)</td>
<td>3.85</td>
<td>0.06</td>
<td>4.16</td>
<td>0.14</td>
<td>6.95</td>
<td>3x</td>
<td>1.08</td>
</tr>
<tr>
<td>Kushima</td>
<td>Ogi79</td>
<td>3 (1)</td>
<td>3.70</td>
<td>0.05</td>
<td>4.01</td>
<td>0.07</td>
<td>6.70</td>
<td>3x</td>
<td>1.08</td>
</tr>
<tr>
<td>Kushima</td>
<td>Ogi80</td>
<td>3 (1)</td>
<td>3.75</td>
<td>0.02</td>
<td>4.13</td>
<td>0.03</td>
<td>6.90</td>
<td>3x</td>
<td>1.10</td>
</tr>
<tr>
<td>Tomakomai</td>
<td>M. sacchariflorus</td>
<td>4 (4)</td>
<td>4.37</td>
<td>0.02</td>
<td>4.94</td>
<td>0.05</td>
<td>8.25</td>
<td>4x</td>
<td>1.13</td>
</tr>
<tr>
<td>Tsukuba</td>
<td>M. sacchariflorus</td>
<td>3 (3)</td>
<td>4.33</td>
<td>0.13</td>
<td>4.94</td>
<td>0.20</td>
<td>8.25</td>
<td>4x</td>
<td>1.14</td>
</tr>
<tr>
<td>Gifu</td>
<td>M. sacchariflorus</td>
<td>3 (3)</td>
<td>4.25</td>
<td>0.01</td>
<td>4.84</td>
<td>0.04</td>
<td>8.08</td>
<td>4x</td>
<td>1.14</td>
</tr>
<tr>
<td>Miyazaki</td>
<td>M. sacchariflorus</td>
<td>3 (3)</td>
<td>4.53</td>
<td>0.13</td>
<td>5.03</td>
<td>0.00</td>
<td>8.40</td>
<td>4x</td>
<td>1.11</td>
</tr>
<tr>
<td>Kushima</td>
<td>M. sacchariflorus</td>
<td>6 (6)</td>
<td>4.83</td>
<td>0.02</td>
<td>5.33</td>
<td>0.08</td>
<td>8.90</td>
<td>4x</td>
<td>1.10</td>
</tr>
<tr>
<td>Tomakomai</td>
<td>M. sinensis</td>
<td>4 (4)</td>
<td>2.76</td>
<td>0.02</td>
<td>3.15</td>
<td>0.02</td>
<td>5.26</td>
<td>2x</td>
<td>1.14</td>
</tr>
<tr>
<td>Tsukuba</td>
<td>M. sinensis</td>
<td>4 (4)</td>
<td>2.87</td>
<td>0.02</td>
<td>3.14</td>
<td>0.09</td>
<td>5.24</td>
<td>2x</td>
<td>1.09</td>
</tr>
<tr>
<td>Gifu</td>
<td>M. sinensis</td>
<td>3 (3)</td>
<td>2.87</td>
<td>0.15</td>
<td>3.10</td>
<td>0.04</td>
<td>5.18</td>
<td>2x</td>
<td>1.08</td>
</tr>
<tr>
<td>Miyazaki</td>
<td>M. sinensis</td>
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<td>2.79</td>
<td>0.19</td>
<td>3.03</td>
<td>0.02</td>
<td>5.06</td>
<td>2x</td>
<td>1.09</td>
</tr>
<tr>
<td>Kushima</td>
<td>M. sinensis</td>
<td>6 (6)</td>
<td>2.88</td>
<td>0.03</td>
<td>3.17</td>
<td>0.09</td>
<td>5.29</td>
<td>2x</td>
<td>1.10</td>
</tr>
</tbody>
</table>

† S.D.: standard deviation
**Discussion**

Dolezel et al. (1992) assessed highly significant differences in plant nuclear DNA contents by using DAPI-FCM and PI-FCM and questioned the reliability of base-preference fluorochromes. Therefore, any initial genome-size report based upon DAPI should be verified using a base-ratio-independent stain. This study revealed comparisons between DAPI-FCM and PI-FCM, indicating that there is a low probability of having missed additional 3x plants in the previous study. The present results show a strong correlation between PI-FCM and DAPI-FCM, indicating that there is a low probability of having missed additional 3x plants in the previous study. The present results show similar AT contents among all ploidy levels of Miscanthus species.

Barow and Meister (2002) reported that although genome size and AT level are not correlated among higher plants as a whole, in most known cases, base composition does not vary strongly among closely related species. This is supported by the close correlation between the real genome size (as estimated with PI) and the apparent genome size (as estimated with DAPI) reported in Lolium perenne (Sugiyama et al. 2002), Cirsium spp. (Bures et al. 2004), and Trifolium spp. (Vizintin et al. 2006). Another study noted that the rather low variability in AT content found in Festuca pallens diploids did not seem to dramatically affect the strong linear DAPI/PI correlation.

Large differences in relative DNA contents were estimated among populations of 4x Miscanthus sinensis with the two dyes. We did not count the chromosome number of Miscanthus sinensis or Miscanthus sacchariflorus; however, previous studies have reported the chromosome number of many Miscanthus sacchariflorus plants as 76, indicating tetraploidy (Hirayoshi et al. 1956; Adati and Shiotani 1962). Few aneuploidy plants were reported in Miscanthus species then aneuploidy would be a factor for variation of relative DNA content (Adati and Shiotani 1962). Rayburn et al. (2009) reported nuclear DNA contents of other ploidy levels of Miscanthus sacchariflorus in the United States, but no other ploidy levels of Miscanthus sacchariflorus were confirmed in Japan besides tetraploid. Therefore, other unknown reasons exist that lead to large differences in relative DNA contents among populations of Miscanthus sacchariflorus. Dwiyanti et al. (2012) reported a putative tetraploid hybrid of Miscanthus sacchariflorus × Miscanthus sinensis from Kushima. If there are some tetraploid hybrids between tetraploid Miscanthus sacchariflorus and diploid Miscanthus sinensis, then these tetraploid hybrids would increase the relative DNA content because the nuclear DNA content of the Miscanthus sinensis genome is 22% larger than that of the Miscanthus sacchariflorus genome (Rayburn et al. 2009). Natural hybridization would be one of the possible reasons for large differences in relative DNA contents among populations of Miscanthus sacchariflorus, but this must be tested using genetic markers in the future. We believe that the results of our previous study regarding relative DNA content in Miscanthus accessions were confirmed. We also think it is possible to estimate the genome size of Miscanthus accessions from DAPI-FCM by using the regression line presented in this study.

**Conclusions**

We conducted a comparison of relative DNA contents estimated with the AT-selective DAPI and intercalary PI in 2x Miscanthus sinensis and 4x Miscanthus sacchariflorus and their 3x hybrids. A strong linear correlation was observed between DAPI-FCM and PI-FCM ($R^2 = 0.99$). This result shows the high reliability of estimations of relative DNA content made in our previous study on Miscanthus accessions.

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**References**


Clifton-Brown J, Lewandowski I, Andersson B,


