Turczaninowia 27, 2: 86–104 (2024)
DOI: 10.14258/turczaninowia.27.2.10
http://turczaninowia.asu.ru

ISSN 1560–7259 (print edition)
TURCZANINOWIA
ISSN 1560–7267 (online edition)

УДК 58.08+575.113+575.224.234.2

Standards in plant flow cytometry: an overview, polymorphism and linearity issues

M. V. Skaptsov[1, 2*], M. G. Kutsev[1, 3], S. V. Smirnov[1, 4], A. V. Vaganov[1, 5], O. V. Uvarova[1, 6], A. I. Shmakov[1, 7]

[1] *Altai State University, South-Siberian Botanical Garden, Lenina Pr., 61, Barnaul, 656049, Russian Federation*

[2] *E-mail: mr.skaptsov@mail.ru; ORCID iD: https://orcid.org/0000-0002-4884-0768*

[3] *E-mail: m_kucev@mail.ru; ORCID iD: https://orcid.org/0000-0003-2284-6851*

[4] *E-mail: serg_sm_@mail.ru; ORCID iD: https://orcid.org/0000-0002-9657-3959*

[5] *E-mail: vaganov_vav@mail.ru; ORCID iD: https://orcid.org/0000-0002-7584-5150*

[6] *E-mail: uwarowa@mail.ru; ORCID iD: https://orcid.org/0000-0003-3318-5163*

[7] *E-mail: alex_shmakov@mail.ru; ORCID iD: https://orcid.org/0000-0002-1052-4575*

** Corresponding author*

Keywords: C-value, DNA content, genome size, plant flow cytometry, ploidy, propidium iodide, reference standards, standardization.

Summary. This article presents a brief review of the main plant species used as standards in flow cytometry. Their linearity was assessed by direct measurements during one year in greenhouse and field conditions. DNA content values were measured at two currently accepted values – based on the human (7.0 pg) and rice (0.795 pg) genomes. It was found that among the DNA content values of human genome-based standards, the DNA content values of *Raphanus sativus* and *Solanum lycopersicum* were not linear. Among the rice genome-based standards, deviations from linearity were found for the genome sizes of *Pisum sativum*, *Vicia faba* and *Allium cepa*. Our direct measurements of DNA content based on rice genome size for commonly used standards such as barley, rye, maize and many others are reported in this paper for the first time. It is found that the non-linearity is mainly related to the genome size value of the primary standard, instrumental variation and polymorphism of the DNA content of the standards. Using multiple standards, some perennial plant species were investigated, which could potentially be used as standards due to low DNA content polymorphism and ease of use. The paper examines in detail the peculiarities of the use of standards and highlights some methodological aspects that ensure the accuracy of data in plant flow cytometry.

Стандарты в проточной цитометрии растений: обзор, вопросы полиморфизма и линейности

М. В. Скапцов, М. Г. Куцев, С. В. Смирнов, А. В. Ваганов, О. В. Уварова, А. И. Шмаков

Алтайский государственный университет, пр. Ленина, д. 61, г. Барнаул, 656049, Россия

Ключевые слова: йодид пропидия, плоидность, проточная цитометрия растений, размер генома, содержание ДНК, стандартизация, эталонные стандарты, C-value.

Аннотация. В статье представлен краткий обзор основных видов растений, используемых в качестве стандартов в проточной цитометрии. Проведена оценка их линейности на основе прямых измерений в тече-

Поступило в редакцию 18.06.2024
Принято к публикации 28.06.2024

Submitted 18.06.2024
Accepted 28.06.2024

ние года в условиях теплицы и открытого грунта. Значения содержания ДНК измерены в двух общепринятых в настоящее время значениях – на основе геномов человека (7,0 пг) и риса (0,795 пг). Выявлено, что среди значений содержания ДНК стандартов на основе генома человека, не линейны значения содержания ДНК у *Raphanus sativus* и *Solanum lycopersicum*. В числе стандартов на основе генома риса выявлены отклонения в линейности для размеров геномов *Pisum sativum*, *Vicia faba* и *Allium cepa*. В работе впервые приведены наши прямые измерения содержания ДНК на основе размера генома риса для часто используемых стандартов, таких как ячмень, рожь, кукуруза и многих других. Выявлено, что нелинейность в основном связана со значением размера генома первоначального стандарта, инструментальными вариациями и полиморфизмом содержания ДНК стандартов. С помощью множественных стандартов исследованы некоторые виды многолетних растений, которые потенциально возможно использовать в качестве стандарта ввиду низкого полиморфизма содержания ДНК и удобства использования. В статье подробно исследованы особенности использования стандартов, а также акцентированно внимание на некоторых методических аспектах, обеспечивающих точность данных в проточной цитометрии растений.

Introduction

Flow cytometry is a modern method of studying the physical properties of particles in a liquid stream activated by a beam of light. Plant flow cytometry is a narrower field of plant research based on the study of the DNA content in isolated plant nuclei. The method is based on a qualitative and quantitative assessment of the fluorescence of isolated plant nuclei stained with non-specific fluorochromes that bind to nucleic acids. The first paper on plant flow cytometry was published by F. Heller (1973), and since the late 1970s the method has been widely used. As rightly stated by Doležel et al. (2007b: 41): "The rise of plant flow cytometry since 1973 is testimony to the impact of a single elementary methodological innovation – the use of a razor blade instead of enzymes for isolation of nuclei".

Standardization is an important aspect of plant flow cytometry, particularly in order to achieve consistent results between laboratories. In the 1980s and 1990s, standardization of plant flow cytometry was particularly challenging due to the parallel development of standardization efforts in different cytometry laboratories. Plant varieties are often used as standards for objective calculations of DNA content. This is due to the relative homogeneity of their genomes and the ability to grow them from seed throughout the year. In addition, the genome changes associated with hybridization processes, which are more common in nature, can be minimized, if the purity of the variety is adequate. However, in some cases, cultivated seed varieties may not be available for research due to factors such as short growing season, seed storage or unavailability of the variety to the researcher. Early studies used chemical extraction and densitometry as main methods, followed by flow cytometry. Plant flow cytometry underwent its most significant development in the 1990s. During this time, several schools of cytometry have developed and several sets of standards have been established. The differences between them are mainly related to the choice of the primary standard (Table 1).

Chronologically, one of the first large-scale studies of plant DNA content was the work of M. Bennett (Bennett, 1972). This was followed by a series of papers on "Nuclear DNA Amounts in Angiosperms" and a set of standards for the analysis of large, medium and small genomes (Bennett, Smith, 1976; Bennett et al., 1982, 2000; Bennett, Leitch, 1995, 1997, 2000, 2001, 2005, 2011). These studies formed the basis of the well-known Kew C-value database. Bennett's standards are based on *A. cepa* (2C = 33.55 pg) as primary standard, with DNA content determined chemically by Van't Hof (1965). Secondary standards were measured either chemically or by densitometry.

Marie and Brown (1993) used chicken red blood cells (CRBC) as the primary standard. Their DNA content was determined biochemically by Galbraith et al. (1983) with a relatively small deviation (2.33 ± 0.22 pg). Arumuganathan and Earle (1991) used the same value for CRBC. Some plant species from their work are also used as standards. Johnston et al. (1999) recalibrated CRBC using flow cytometry against *Tetrodes* sp. and obtained a value of 3.01 pg.

The most widely used are the Doležel's standards (Doležel et al., 1992, 1994, 1998) based on human male leukocytes (HML, 2C = 7 pg) used as the primary standard (Tiersch et al., 1989). The values of the Doležel's standards were obtained in a cascade manner: *Pisum sativum* and *Zea mays* were measured directly with HML, *Solanum lycopersicum* with *Z. mays*, *Raphanus sativus* with *S. lycopersicum* (Doležel, 1992). And vice versa for large standards: *Vicia faba* was measured on *P. sativum*, *Allium cepa* on *V. faba*. *Glycine max* was measured by HML in a separate study (Doležel, 1994). In Doležel et al. (1998), the data for *V. faba* and *A. cepa* were slightly

adjusted and *Hordeum vulgare* and *Secale cereale* were measured with *P. sativum*. However, according to the original source of Tiersch et al. (1989), the value for human DNA content has a very high deviation (7 ± 1.65 pg) and is the average value of DNA content obtained in 11 different studies. Probably, the most accurate method for determining genome size is whole genome sequencing. At present, the size of the human genome determined by sequencing is 6110 Mbp (6.25 pg) (Nurk et al., 2022).

Table 1

The most widely known sets of standards have been established for the study of DNA content

Standards	**Primary standards**					
	***Allium cepa* (33.55 pg)[1]**	***Hordeum vulgare* (11.12 pg) and CRBC (3.01 pg)[2]**	**CRBC (2.33 pg)[3]**	**HML (7.0 pg)[4]**	***Pisum sativum* (8.76 pg)[5]**	***Oryza sativa* (0.795 pg)[6]**
Oryza sativa L.	'IR36' (1.01 pg)					'Nipponbare' (0.795 pg)
Sorghum bicolor (L.) Moench		'Pioneer' (1.74 pg)				
Petunia hybrida (Hook.) Vilm.			'PxPc6' (2.85 pg)			
Vigna radiata (L.) R. Wilczek	'Berken' (1.06 pg)					
Raphanus sativus L.				'Saxa' (1.11 pg)		0.997 pg
Solanum lycopersicum L.			'Mont faveť 63/5 (2.01 pg)	'Stupické polní ranéʼ (1.96 pg)	1.90 pg	1.735 pg
Glycine max (L.) Merr.				'Polanka' (2.50 pg		2.077 pg
Bellis perennis L.					3.46 pg	3.159 pg
Zea mays L.	'W64A' (5.47 pg)			'CE-777' (5.43 pg)	2.30 pg	
Pisum sativum L.	'Minerva Maple' (9.73 pg)	'Minerva Maple' (9.56 pg)	'Express long' (8.37 pg)	'Ctirad' (9.09 pg)		8.018 pg
Hordeum vulgare L.	'Sultan' (11.12 pg)	'Sultan' (11.12 pg)		'Ditta' (10.43 pg)	5.47 pg	
Secale cereale L.	'Petkus Spring' (16.57 pg)			'Dankovske' (16.19 pg)	8.76 pg	
Vicia faba L.	PBI. inbred line (26.66 pg)	'GS011' (26.66 pg);		'Inovec' (26.90 pg).	26.92 pg	23.796 pg
Allium cepa L.	'Ailsa Craig' (33.55 pg)	'Ailsa Craig' (33.55 pg)		'Alice' (34.89 pg)		30.745 pg
Triticum aestivum L.	'Chinese Spring' (34.64 pg)		'Triple Dirk' (30.9 pg)			

Notes: [1] Bennet, Leitch, 1997; [2] Johnston et al., 1999; [3] Marie, Brown, 1993; [4] Doležel et al., 1992, 1994, 1998; [5] Kubešová et al., 2010, the same varieties; [6] Veselý, 2011; Veleba, 2017; Šmarda, 2014, the same varieties.

Based on the spread of sequencing methods, the Doležel's set of standards was further developed. The Doležel's standards were re-measured against *Oryza sativa* (Veselý et al., 2012; Šmarda et al., 2014; Veleba et al., 2017), which at that time was considered to be fully sequenced with a genome size of 777.64 Mbp (0.795 pg) (International Rice Genome Sequencing Project, 2005). According to the most recent data, using a hybrid strategy of long read and short read sequencing, a value of 771.4 Mbp was obtained, which is close to the previous data (Shang et al., 2023). Thus, the values based on *O. sativa* are currently the most accurate and closest to the real values. Veselý et al. (2011) measured the DNA content of *R. sativus* and *S. lycopersicum* directly using *O. sativa,* and *B. perennis* using *S. lycopersicum*. Veleba et al. (2017)

additionally measured *G. max* using *S. lycopersicum*. The 2C values of *P. sativum, V. faba* and *A. cepa* were recalculated using the Doležel et al. (1992) ratios. Some new standards were also proposed, including *Carex acutiformis* Ehrh., *Epipremnum aureum* (Linden et André) G. S. Bunting and *Haemanthus albiflos* Jacq.

Currently, many authors agree that in order to be able to recalculate the obtained data in future works, it is important to use a limited number of standards in Doležel's or Veselý's values (Temsch et al., 2022). We also support this statement to ensure data stability between laboratories.

Therefore, the standards in use today were determined by different methods, at different times, using different equipment and different primary standards. Often, DNA content values are an average of data from several studies. This could be a possible explanation for the non-linearity of the results. As J. Suda writes in his PhD thesis (2004: 31 p.): "If more internal standards are necessary (to span the range of DNA amounts), the secondary standard must be calibrated against the primary one (it is not a good idea to use tabulated values)". In this regard, the task of this study is to evaluate the linearity of the most common standards, review the lines of standards, features of their use, and also evaluate some methodological aspects. We found similar problems with linearity when working with small genomes using Doležel's values. When measuring the DNA content of *Ficus benjamina* L. using *G. max* (2.5 pg), a value of 1.07 pg was obtained (Skaptsov et al., 2016). However, when measured using *R. sativus* (1.11 pg), a value of 0.9 pg was obtained. This led us to investigate the linearity of the most commonly used standards in more detail.

Materials and methods

We have carried out more than 2,000 measurements over a period of 12 months. The plants were grown in the open field and under greenhouse conditions. The species and varieties of plants used are listed in Supplement 1.

In our article, the 2C data are mostly given in two values: the classical Doležel's values based on the human genome size (7.0 pg) and the Veselý's values based on the *O. sativa* genome size (0.795 pg); further in the text HML-based and *O. s.*-based (Doležel et al., 1992; Veselý et al., 2012; Šmarda et al., 2014; Veleba et al., 2016).

DNA content was determined by flow cytometry with propidium iodide (PI) staining. Fresh leaves were used as samples. Internal standardization was used (samples and standards co-chopped together). One-step and two-step protocols were used, with LB01, Tris-$MgCl_2$ and Otto buffers (Doležel et al., 2007a).

In the one-step protocol, samples were co-chopped with the standard using a sharp razor blade in 1 ml Tris-$MgCl_2$ buffer (Prosser et al., 1995) or LB01 buffer (Doležel et al., 1992) supplemented with PI (50 µg/ml), RNase (10 µg/ml) and 12 mM sodium thiosulfate. The nuclear suspension was filtered through a nylon filter with a pore size of 50 microns and incubated at room temperature for 15 minutes.

In the two-step protocol, samples were co-chopped with standard in 0.5 ml modified Otto I buffer (0.1 M citric acid, 0.5 % Triton X-100), then filtered through a nylon filter and incubated on ice for at least 10 minutes (up to several hours). The sample was centrifuged at 200g for 5 minutes and the supernatant removed and discarded. The pellet was resuspended in 200 µl of Otto I buffer. To this suspension, 200 µl of modified Otto II buffer (0.4 M $Na_2HPO_4 \times 2H_2O$, 100 µg/mL PI, 20 µg/mL RNase, 24 mM sodium thiosulphate) was added and incubated for 30 minutes at room temperature.

Analyses were performed on a Cytoflex (Beckman Coulter, USA) and CyFlow PA (Partec, Germany) cytometer. Peaks with at least 1000 nuclei and a CV of less than 3 % were used for analysis. Histograms were visualised and processed using CytExpert software (Beckman Coulter, Inc.). Statistical data were calculated using XLStat (Addinsoft). Calculations of the 2C value were primarily performed using a single standard approach, but with two or more internal standards. When samples were within the range of the standards, the regression line approach was used (Yokoya et al., 2000; Temsch et al., 2021).

Notes: 1. A sodium thiosulfate solution (12 mM) has been employed as an alternative to mercaptoethanol (toxic and unpleasant odour), which has been combined with polyvinylpyrrolidone (PVP) (1 %) when working with woody plants. It is also possible to use DTT up to 10 mM, 10 mM sodium metabisulphite or PVP up to 2 % as an alternative (Greilhuber et al., 2007; Loureiro et al., 2007). However, our experience has shown that PVP is better used in combination with sodium metabisulphite, DTT or sodium thiosulphate. 2. Typically, 1–2 minutes are required to stain the sample. Incubation is required for RNA digestion. Incubation for 15 minutes at 37–42 °C may be helpful for difficult samples with metabolites, high RNA content or poor histograms with noise and high CV. 3.

Triton X-100 is used instead of Tween 20 to improve the isolation of nuclei in Otto I buffer. 4. There are many variations of Otto buffers (Otto, 1990; Doležel et al., 2007a; Šmarda et al., 2019): with and without centrifugation; original or acidified; different ratios of Otto I-II (1:1 or 1:2). In our case, the volumes are selected for cytometers with a peristaltic sampler (Cytoflex). Displacement samplers on Sysmex (formerly Partec) instruments require a large sample volume (1 ml to 1 ml or 0.5 ml to 1 ml). It is important that the ratios are maintained. 5. The concentrations of the additional components of the Otto II buffer are given on the basis of an Otto I-II ratio of 1:1, and the final concentration will be standard (50 µg/mL PI, 10 µg/mL RNase, 10 mM sodium thiosulphate). 6. The use of $Na_2HPO_4 \times 12H_2O$ may cause problems with buffer crystallization. Dihydrate is used in the original article. 6. Otto buffers are suitable for live material with low levels of metabolites and RNA. This buffer is suitable for limited plant groups. The results are very stable between measurements with low CVs of peaks on the histograms. With Otto I buffer, isolated nuclei can be stored on ice or at room temperature for several hours. Since citric acid preserves nucleic acids and the pH is not optimal for nucleases, it is convenient to prepare a large number of samples in the first step and store them until Otto II is added. In any case, the choice of buffer is very species specific.

Results and discussion

Three different buffers were used for the measurements: Tris-$MgCl_2$, LB01 and Otto. Between Tris-$MgCl_2$ and LB01 there was very little difference. In most cases the data were slightly different in Otto. In LB01 and Otto buffer, this appears to be due to the unequal increase in fluorescence of the nuclei of different species. For example, the DNA content of pea is 2.02-fold higher than that of parsley in Tris-$MgCl_2$and 2.05-fold higher in LB01 and Otto buffer. The only difference between Otto and LB01 buffers was a higher level of fluorescence, as reported by Loureiro et al. (2006). *R. sativus* was an exception, so the ratios for it are given in three different values (Table 1). The ratio of *R. sativus* to *S. lycopersicum* in Otto buffer is similar to Veselý's values, as Otto buffer was also used in their experiment. *R. sativus* samples have a high level of endopolyploidy and similar deviations were observed in active growth and heavily pubescent leaves, which may be due to the presence of metabolites or environmental factors.

As seen in Table 2, *R. sativus*, *S. lycopersicum*, *G. max* and *B. perennis* standards at Veselý's value are linear among themselves and close to published ratios. *P. sativum*, *V. faba* and *A. cepa* are linearly related to each other. In the measurement of *P. sativum* via *S. lycopersicum, G. max*, and *Bellis perennis.*, the 2C values were lower than 8.018 pg.

There is a similar situation with the Doležel's values. *R. sativus* and *S. lycopersicum* are linear but only in the Otto buffer. All other standards higher from *G. max* to *A. cepa* are also linear (*Z. mays* in the value 5.72 pg, Doležel et al., 1992). When comparing the measurements of *S. lycopersicum* with *P. sativum* and *S. lycopersicum* with *G. max*, there are differences from the published values. Also, when comparing the ratios of Veselý and Doležel, it is clear that the theoretical ratios between the values of *R. sativus* to *S. lycopersicum* (0.575/0.566) and *G. max* to *S. lycopersicum* (0.835/0.784) differ. Our data are more similar to Veselý ratios in these standards, while in the case of *G. max* to *P. sativum* our data are closer to Doležel's values.

The measurement with several standards in the Doležel's value, using *P. sativum* as the primary standard, gave data close to the published values (within 3 %) for all standards except *S. lycopersicum* and *R. sativus* (see Table 2). Small differences were observed for *Z. mays*. The Veselý's value was based on *S. lycopersicum*. Highly linear results and values close to those published were obtained for *R. sativus* (in Otto buffer), *S. lycopersicum*, *G. max*, *B. perennis* and *E. aureum*. *P. sativum*, *V. faba* and *A. cepa* showed values lower than those published.

In Table 3, some less commonly used standards were also measured for the first time in the HML-based and *O. s.*-based values. These included *Petroselinum crispum* (Mill.) Fuss, *Petunia hybrida* (Hook.) Vilm., *Solanum pseudocapsicum* L., *Chlorophytum comosum* (Thunb.) Jacques, *Allium fistulosum* L., and species that could potentially be used as standards – *Euonymus japonicus* (E. j.), *Solanum tuberosum* L., *Allium tuberosum* Rottler ex Spreng. Some of the Doležel's standards were directly measured in the *O. s.*-based values, such as *H. vulgare*, *S. cereale*, etc.

On the basis of the data obtained, the linearity of the standards was slightly adjusted (see Table 4, Fig. 1). According to Table 4, it is necessary to adjust the DNA content values for *R. sativus* and *S. lycopersicum* in the HML-based value. The other standards are in agreement.

Table 2

The ratios are based on the known values of the DNA content of the standards and on our direct measurements based on original values

Theoretical ratios based on *Oryza sativa* (0.795 pg)[2]		**Theoretical ratios based on HML (7.0 pg)**[1]		**Directly measured ratios (this work)**	**Average 2C, pg (*O. s.*-based)**	**Average 2C, pg (HML-based)**
Standard (2C, pg)	**Ratios**	**Standard (2C, pg)**	**Ratios**			
R. s. (0.997)/S. l. (1.735)	**0.575**	R. s. (1.11)/S. l. (1.96)	**0.566**	Mean 0.587 Otto 0.578 1step 0.598	**1.698 (2.20 %)** 1.724 (0.66 %) **1.668 (3.89 %)**	**1.890 (3.70 %)** 1.920 (2.14 %) **1.857 (5.28 %)**
S. l. (1.735)/B. p. (3.159)	0.549			0.553	3.136 (0.72 %)	
S. l. (1.735)/G. m. (2.077)	**0.835**	S. l. (1.96)/G. m. (2.5)	**0.784**	0.834	2.081 (0.19 %)	**2.351 (5.97 %)**
G. m. (2.077)/B. p. (3.159)	0.657			0.666	3.119 (1.27 %)	
S. l. (1.735)/P. s. (*8.018*)	0.216	S. l. (1.96)/P. s. (9.09)	0.216	*0.230*	**7.549 (5.85 %)**	**2,089 (6.59 %)**
G. m. (*2.077*)/P. s. (8.018)	**0.259**	G. m. (2.5)/P. s. (9.09)	**0.275**	*0.269*	**7.790 (3.86 %)**	2.449 (2.03 %)
B. p. (3.159)/P. s. (*8.018*)	0.394			0.407	**7.761 (3.20 %)**	
P. s. (8.018)/V. f. (23.796)	0.337	P. s. (9.09)/V. f. (26.9)	0.338	0.334	24.003 (0.86 %)	27.21 (1.15 %)
V. f. (23.796)/A. c. (30.745)	0.774	V. f. (26.9)/A. c. (34.89)	0.771	0.771	30.860 (0.37 %)	34.886 (0.01 %)
P. s. (8.018)/H. v. (9.195[5])	0.872	P. s. (9.09)/H. v. (10.43)	0.872	0.860	9.323 (1.38 %)	10.570 (1.33 %)
P. s. (8.018)/S. c. (14.292[5])	0.561	P. s. (9.09)/S. c. (16.19)	0.561	0.550	14.578 (1.96 %)	16.520 (2.03 %)
Z. m. (5.063[3])/P. s. (8.018)	0.631	Z. m. (5.433)/P. s. (9.09) Z. m. (5.72)/P. s. (9.07)	0.598 0.631	0.623	**4.805 (5.09 %)**	**5.660 (4.18 %)** 5.660 (1,04 %)
S. l. (1.735)/Z. m. (5.063[3])	0.343	S. l. (1.96)/Z. m. (5.433) S. l. (1.96)/Z. m. (5.72)	0.361 0.343	0.371	**4.680 (8.19 %)**	5.287 (2.77 %) 5.287 (8.20 %)
O. s. (0.795)/R. s. (0.997)	0.797			0.797	0.998 (0.09 %)	
A. t. (0.304[4])/O. z. (0.795)	0.382			0.376	0.299 (1.64 %)	

Notes: Measurements were performed in a cascade in pairs using a single standard. The 2C values we obtained with a deviation of more than 3 % and the differences between the theoretical ratios of Doležel and Veselý are highlighted in **bold**. When calculating 2C, the logic of the measurements in the Veselý set (from small to large genomes) and in the Doležel set (from *P. sativum*) is preserved. Deviation from published data is indicated in parentheses.

[1] Doležel et al., 1992, 1994, 1998; [2] Veselý et al., 2012; Šmarda, 2014; Veleba, 2017; [3] Recalculated by us on the basis of the ratios of Doležel et al., 1992; [4] Šmarda et al., 2019; [5] Recalculated by us on the basis of the ratios of Doležel et al., 1998.

A. t. – *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia; O. s. – *Oriza sativa* var. *japonica* unknown var.; R. s. – *Raphanus sativus* 'Saxa'; S. l. – *Solanum lycopersicum* 'Stupicke polni rane'; G. m. – *Glycine max* 'Polanka'; B. p. – *Bellis perennis* unknown varieties; Z. m. – *Zea mays* 'CE777'; P. s. – *Pisum sativum* 'Ctirad'; H. v. – *Hordeum vulgare* 'Ditta'; S. c. – *Secale cereale* 'Tatyana'; V. f. – *Vicia faba* 'Inovec'; A. c. – *Allium cepa* 'Stuttgarter Riesen'.

However, standards with larger genomes, such as *P. sativum*, *V. faba* and *A. cepa*, need to be adjusted in the Veselý's value. Our direct measurements showed that the genomes of these standards differ by about 3–4 %.

Within these groups, the standards are linear and the data obtained from the measurement of third-party samples have a deviation of less than 3 %. The application of the HML-based and *O. s.*-based values and the corrected values are given in the Supplement on the journal's website. The standards in the published and new values were compared in pairs with each other and with third party sources (see Supplement 1 on the journal's website). It was found that when the non-linear standards in the classical values were used for measurements, the coefficient of variation (CV) was usually higher than 3 %. However, when the corrected values were used, the CV did not exceed 2–3 %.

Table 3

DNA content of the studied plant species based on our direct measurements with multiple standards in HML-based and *O. s.*-based values

Species	**2C ± RMSE, pg (R^2/ MAPE, %), HML-based (7.0 pg)**	**2C ± RMSE, pg (R^2/ MAPE, %), *O. s.*-based (0.795 pg)**	**N**	**Standards**
Arabidopsis thaliana (A. t.)	*0.366 ± 0.006 (1.50)*	*0.304 ± 0.004(1.45)*	9	E. j., F. b., R. s., S. l.
Raphanus sativus (R. s.)	*1.225 ± 0.026 (2.14 %)* *Otto 1.201* *One-step 1.246*	*1.002 ± 0.021 (2.06 %)* *Otto 0.997* *One step 1.04*	101	S. l., G. m.
Solanum lycopersicum (S. l.)	*2.077 ± 0.034 (1.62 %)*	1.735*	171	G. m., B. p., P. c., P. s.
Glycine max (G. m.)	2.492 ± 0.117 (0.99/1.56)	2.091 ± 0.100 (0.99/1.59)	117	S. l., B. p., P. c. P. s.
Bellis perennis (B. p.)	3.732 ± 0.06 (1.00/0.96)	3.147 ± 0.053 (1.00/0.86)	18	R. s., S. l., G. m., P. c., P. s.
Petroselinum crispum (P. c.)	4.508 ± 0.075 (0.999/1.021)	3.806 ± 0.064 (0.99/1.04)	130	S. l., G. m., B. p., P. s.
Zea mays (Z. m.)	5.646 ± 0.053 (1.00/0.75)	4.778 ± 0.05 (1.00/0.83)	92	P. c., P. s., S. l., G. m., B. p., E. a.
Pisum sativum (P. s.)	9.09*	*7.717 ± 0.110 (1.43 %)*	201	S. l., G. m., B. p.
Epipremnum aureum (E. a.)	9.507 ± 0.118 (1.00/0.87)	8.115 ± 0.098 (1.00/0.86)	18	S. l., G. m, B. p, P. c., V. f., A. c.
Hordeum vulgare (H. v.)	10.55 ± 0.023(1.00/0.24)	8.975 ± 0.026(1.00/0.27)	19	B. p, P. c., P. s., V. f., A. c.
Secale cereale (S. c.)	16.312 ± 0.256 (1.00/1.03)	13.966 ± 0.207(1.00/0.89)	15	Z. m., P. s., H. v., V. f., A. c.
Vicia faba (V. f.)	*27.228 ± 0.182 (0.67 %)*	*23.075 ± 0.175 (0.76 %)*	37	P. s., E. a.
Allium cepa (A. c.)	*35.018 ± 0.293(0.84 %)*	*29.920 ± 0.235 (0.79 %)*	31	P. s., V. f.
Rarely used standards or potential standards				
Euonymus japonicus (E. j.)	*0.569 ± 0.006 (1.12)*	*0.688 ± 0.007 (1.09)*	9	F. b., R. s., S. l.
Ficus benjamina (F. b.)	1.069 ± 0.014 (1.32)	0.888 ± 0.009 (0.97)	12	R. s., S. l., G. m., B. p.
Petunia hybrida (P. h.)	2.598 ± 0.043 (1.00/0.85)	3.086 ± 0.049(1.00/0.76)	18	R. s., S. l., B. p., P. c., P. s.
Solanum pseudocapsicum (S. p.)	2.835 ± 0.035(1.00/0.55)	2.386 ± 0.03 (1.00/0.54)	15	S. l., B. p., P. c., Z. m., P. s.
Solanum tuberosum (S. t.)	3.800 ± 0.034(1.00/0.62)	3.200 ± 0.032(1.00/0.80)	18	S. l., G. m., P. c., Z. m., P. s.
Chlorophytum comosum (Ch. c.)	23.993 ± 0.22 (1.00/0.63)	20.553 ± 0.191 (1.00/0.635)	9	P. s., V. f., A. c.
Allium fistulosum (A. f.)	25.004 ± 0.150(1.00/0.661)	21.426 ± 0.131 (1.00/0.597)	15	E. a., P. s., H. v., V. f., A. c.
Allium tuberosum (Al. t.)	*64.540 ± 0.343(0.53 %)*	*55.393 ± 0.289 (0.52 %)*	9	V. f., S. c., A. f.
Haemanthus albiflos (H. a.)	*72.725 ± 0.855 (1.18 %)*	*62.373 ± 0.722 (1.16 %)*	9	V. f., A. f., Al. t.

Notes: * primary standard; The calculated average 2C values, standard deviation (SD) and coefficient of variation (CV) are highlighted in *italic*; because the measured sample is outside the standard range, the regression line is not used.
RMSE – Root mean square deviation (analogue SD); MAPE – mean absolute percentage error (analogue of CV); N – number of measurements.
The measurement logic is such that each newly measured standard is used in the measurement of the next standard if there is no peak overlap. The new value is used if the DNA content differs by more than 3 %. For measurements and calculations R. s., S. l., G. m., B. p., E. a. in *O. s.*-based value are used without changes, new 2C values were used for P. s. (7.717 pg), V. f. (23.075), A. c. (29.92). In HML-based value without changes were used G. m., P. c., P. s., H. v., V. f., A. c., new 2C values were used for R. s. (1.201/1.246 pg), S. l. (2.077 pg).
Pairwise ratios are presented in Supplement 1 on the journal's website.

Table 4

The values of the standards based on direct cytometry measurements are linear among themselves, in two values, the HML-based value and *O. s.*-based value.

	A. t.	R. s.	S. l.	G. m.	B. p.	P. c.	Z. m.	P. s.	H. v.	V. f.	A. c.
2C, pg, HML-based	**0.366**	**1.225**	**2.077**	2.5	**3.732**	4.5[c]	**5.646**	9.09	10.43	26.9	34.89
2C, pg, *O. s.*-based	0.304[a]	0.997	1.735	2.077	3.159	**3.806**	**4.778**	**7.717**	**8.975**	**23.075**	**29.92**
Rarely used standards or potential standards											
E. j.	F. b.	P. h.	S. p.	S. t.	E. a.	S. c.	Ch. c.	A. f.	Al. t.	H. a.	
0.569	1.07[d]	**2.598**	**2.835**	**3.8**	**9.507**	16.19	24.14[b]	**25.004**	**64.54**	**72.905**	
0.688	**0.888**	**3.086**	2.414*	**3.2**	7.991	**13.966**	**20.553**	**21.426**	**55.393**	**62.531**	

Notes: Explanation of species abbreviations in Table 3. New DNA content data, corrected or examined for the first time in two values, are highlighted in **bold**. Regular font data, previously published and close to our measurements (with a deviation of less than 3 %).
* Temsch et al., 2022, unpublished value of P. Šmarda; [a] Šmarda et al., 2019; [b] Hornych et al., 2019; [c] Obermayer et al., 2002; [d] Skaptsov et al., 2016. *Oryza sativa* in the Doležel's value, recalculated by the Veselý's values with *Raphanus sativus* and *Solanum lycopersicum*, is 0.955 pg.
Based on unaltered *O. s.*-based values, these standards will have a DNA content of: P. c. – 3.90 pg, S. c. – 14.511 pg, Ch. c. – 21.355 pg, A. f. – 22.262 pg; Al. t. – 57.554 pg, H. a – 64.97 pg.

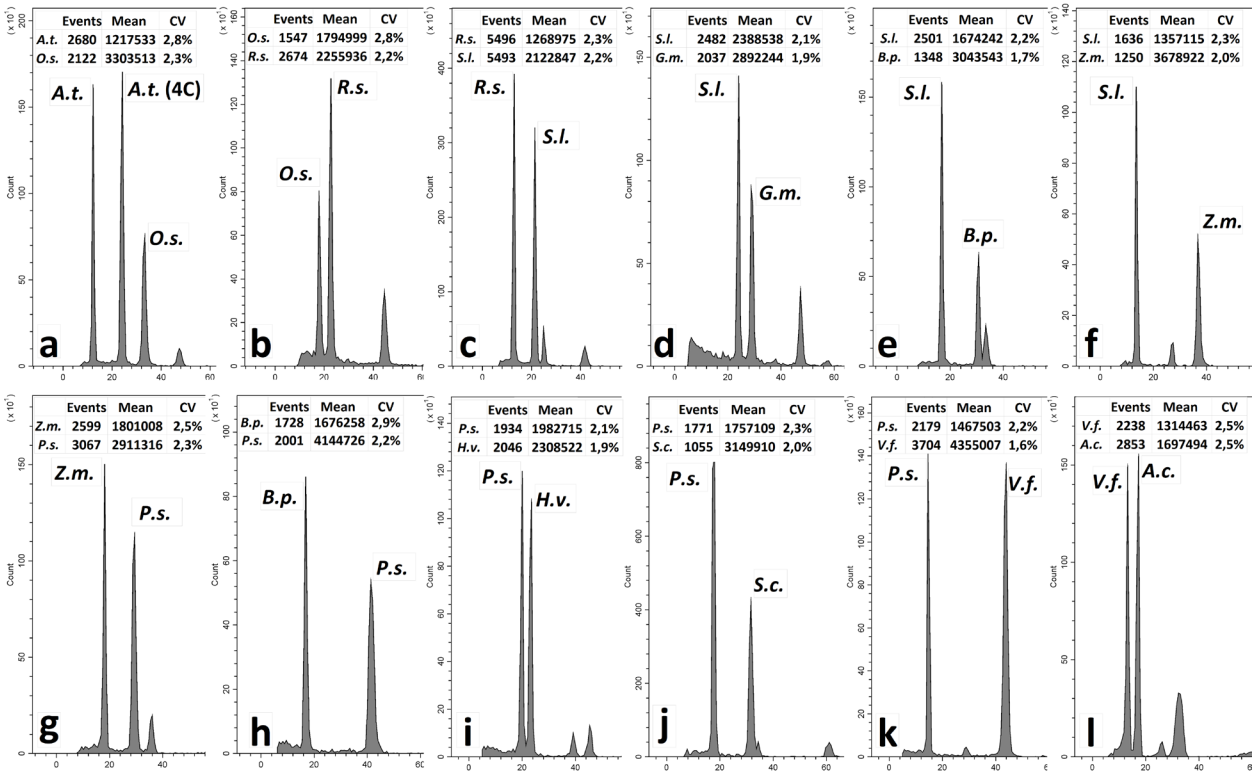

Fig. 1. Examples of ungated histograms for the most commonly used standards. Linear scale. Internal standardization: a – *Arabidopsis thaliana* and *Oryza sativa*; b – *Oryza sativa* and *Raphanus sativus*; c – *Raphanus sativus* and *Solanum lycopersicum*; d – *Solanum lycopersicum* and *Glycine max*; e – *Solanum lycopersicum* and *Bellis perennis*; f – *Solanum lycopersicum* and *Z. mays*; g – *Z. mays* and *Pisum sativum*; h – *Bellis perennis* and *Pisum sativum*; i – *Pisum sativum* and *Hordeum vulgare*; j – *Pisum sativum* and *Secale cereale*; k – *Pisum sativum* and *Vicia faba*; l – *Vicia faba* and *Allium cepa*.

Based on the direct measurements provided in Supplement 1, it is clear that, when using published values, the coefficient of variation (CV) of the studied samples often exceeds 3 %. When measuring samples with a medium genome (between *S. lycopersicum* and *P. sativum*), those measured using *P. sativum* and *V. faba* have a higher 2C value than those studied with *S. lycopersicum* and *B. perennis*. On the other hand, samples analysed with *R. sativus* and *S. lycopersicum* have a lower DNA content than those analyzed with *G. max*, *P. crispum* and *P. sativum*.

During our research, carried out in different seasons and under different conditions throughout the year, we identified some problematic standards. It is better to avoid using radish and soybeans in experiments and use another standard instead. This is because of their different behavior when working with different buffers, as well as the polymorphism of DNA content in different tissues and organs. Alternatively, one can constantly check the linearity with other standards to ensure accuracy.

In most cases *R. sativus* should be used at different 2C values: 0.997/1.04 pg in a two-step protocol and 1.201/1.246 pg in a one-step protocol. The linearity of *R. sativus* should also be checked with *S. licopersicum* or other standards. There were also occasional problems with *B. perennis*, associated with not always high-quality histograms and polymorphism between individuals. Almost all large standards such as *P. sativum*, *H. vulgare*, *V. faba* and *A. cepa* are very stable. Among the medium-sized standards, parsley showed low levels of DNA variation and high linearity compared to *P. sativum*. Instead of *R. sativus* and *G. max*, small standards can be used, such as *O. sativa* 'Nipponbare', *S. lycopersicum* or other standards with a small genome that we have measured (*S. pseudocapsicum*, *F. benjamina*, etc.). However, *S. lycopersicum*, especially very young leaves in some cases, can also show variation.

Discussion

There are many examples of standards being recalibrated. These include both individual standards (Leong-Skornicková et al., 2007; Suda et al., 2007) and entire sets of standards (Kubešová et al., 2010; Praça-Fontes et al., 2011; Veselý et al., 2012; Šmarda et al., 2014; Veleba et al., 2016). A careful examination of the original sources shows that in most cases it is a question of replacement of the primary standard or its recalibration. In this case, however, it is not the DNA content values that you need to focus on but rather the ratios between the different standards. Although the values may vary, the ratios may remain the same or similar. In most cases, the Otto buffer was used in these works. Based on our results, some standards behave differently in the Otto buffer and in the one-step protocol.

O. s.-based standards

Based on our measurements, the *O. s.*-based values lose linearity at the level of *P. sativum*. According to Veselý et al. (2012), Doležel's standards were measured with *O. sativa* (0.795 pg) using the single standard method or the cascade method. However, it is clear that only *R. sativus*, *S. lycopersicum* and *G. max* were directly measured among the Doležel's standards when analyzing the works of Veselý et al. (2012), Veleba et al. (2016) and Šmarda et al. (2014). In addition, *B. perennis*, *C. acutiformis* and *E. aureum* have also been measured directly. If we recalculate using the ratios of *R. sativus* (1.11 pg), the DNA content for these species is lower than the HML-based, 1.93 and 2.34 pg, respectively. This is confirmed by our data. The rest of the Doležel's standards were recalculated on the basis of the ratios of *S. lycopersicum* to *Z. mays* and *Z. mays* to *P. sativum* from the article of Doležel et al. (1992).

The logic of this recalculation is quite clear, since the recalculation results in data are very close to the size of the sequenced human genome. That is, in fact, the first half of the standards of Veselý et al. (2012) are based on *O. sativa* and the second half on human DNA content data. The use of standards in the *O. s.*-based studied directly (*O. sativa*, *R. sativus*, *S. lycopersicum*, *G. max*, *B. perennis*, *E. aureum*) leads to very accurate results between laboratories, for example, *Arabidopsis thaliana, S tuberosum* and many other species we studied have 2C values very close to the values of Šmarda et al. (2019). In practice, using *O. s.*-based values without changes, especially when working in an Otto buffer, can be justified, but the variations will be in the range of 3–5 %. The variation will be even smaller, when two or more standards are in use or when a single standard is used for all samples in a study. Also based on these data, it is clear that the cytometry data are not exactly linear with the sequencing data. This becomes especially clear on the basis of published data and our direct measurements of *Arabidopsis* (below).

Slight differences in the ratios of e.g. *P. sativum* to *V. faba* and *A. cepa* are due to the fact that in the work of Doležel et al. (1992) the DNA content of *P. sativum* and *A. cepa* was 9.07 pg and 34.76 pg, which was used for recalculation. Thus, what is directly measured agrees with our results, and what

is recalculated has a higher DNA content than what we found. The linearity of *S. lycopersicum*, *G. max* and *B. perennis* with *E. aureum*, since *E. aureum* was measured directly, also confirms this pattern.

HML-based standards

As can be seen from our results, *R. sativus* and *S. lycopersicum* standards are not linear with respect to all other standards in HML-based values. Our results of DNA content and the ratio of *S. lycopersicum* (2.077 pg) to *G. max* (2.5 pg) with direct measurement is 0.831, which is close to the ratios of Veleba et al. (2016). If we use the ratio of *G. max* to *S. lycopersicum* (1.735/2.077 = 0.835), the values in Doležel are 2.088/2.5. The values obtained by Kron and Husband (2012) are also close, as they obtained a 2C value for *S. lycopersicum* (2.12 pg) on the basis of *G. max*. Kubešova et al. (2010) gave values for *G. max* and *S. lycopersicum* of 1.90 and 2.3 pg, respectively, which, when calculated by the ratio (1,9/2,3 = 0,826), are also close to our results (2,10/2,5 pg). A similar value for tomato (2.11 pg) was obtained in Leong-Skornicková et al. (2007), where *G. max* (2.5 pg) was also used as a primary. The linearity of *G. max* with *P. sativum* is related to the fact that it was later measured directly with HML (Doležel et al., 1994). Our data on the ratio of *Z. mays* to *P. sativum* (5.646/9.09 pg) are similar to those reported by Suda et al. (2007). However, J. Suda used *Z. mays* (5.43/8.85 pg) as the primary standard, so if *Z. mays* and *P. sativum* were converted, the ratio would be 5.58/9.09 pg. These values confirm our findings on the non-linearity of *R. sativus* and *S. lycopersicum* compared to Doležel's large genome standards.

The ratios of *P. hybrida* 'PxPc6' (2.85 pg), *S. lycopersicum* 'Mont Favet' (2.01 pg) and *A. cepa* (32.7 pg) to *P. sativum* 'Express long' (8.37 pg) were calculated according to Marie and Brown (1993) and yielded ratios that were found to be in close agreement with the data obtained in this study. Even though we used the 'Ctirad' and 'Stupice' varieties. Thus, the DNA content of *P. sativum* according to Marie and Brown (1993) is lower than the values of the standards of other authors. This is because the measurement was carried out from standards with a small genome to standards with a large genome. On the basis of the above, the ratios between the species studied are close regardless of the cultivar.

In Praça-Fontes et al. (2011), the ratios are close to our data for *S. lycopersicum*–*G. max*, *A. cepa*–*V. faba*, *A. thaliana*–*R. sativus*. The deviations of other ratios are probably related to the use of Otto buffer only, since a disproportionate increase in fluorescence was observed for some standards. And, as we have noted, may be related to differences in DNA content between leaves for some standards such as *G. max*, *R. sativus*, *S. lycopersicum*, or, as is sometimes suggested, to environmental factors.

In addition to technical reasons related to the level of equipment in those years, the reason for the loss of linearity in *R. sativus S. lycopersicum* may also be related to the characteristics of *Z. mays*. It is possible that the difference between the linearities of *R. sativus* to *S. lycopersicum* and *Z. mays* and above is due precisely to the polymorphic nature of the *Z. mays* genome, for which significant variations of 5 to 6.8 pg are known due to additional B chromosomes (Rosato et al., 1998; Rosado et al., 2009). To the same Suda and Leitch (2010) also pointed out but associated the variability of *Z. mays* with transposons.

Many standards obtained from more than one paper may differ. This is due to differences in cytometers, nuclear isolation methods or measurement approaches. Many values are averages. Again, *Z. mays* is an example of averaging. The DNA content was initially calculated to be 5.72 pg relative to HML, the same as for *P. sativum* (9.07 pg) (Doležel et al., 1992). The *Z. mays* value was subsequently revised to 5.433 pg, but this value is not included in the Doležel et al. (1998) paper. It appears in Lysák, Doležel (1998) and later in Doležel, Bartos (2005). It should be noted that the value of 5.43 pg was obtained using *P. sativum* (9.09 pg) and is the average of two values obtained on two different lamp cytometers (5.54 pg and 5.33 pg, Doležel et al., 1998). However, the average of four measurements on lamp and laser cytometers is 5.67 pg and is close to our direct measurements. Interestingly, *Z. mays* is linear at 5.43 compared to *S. lycopersicum* at 1.96 pg when measured directly. Often there are single standards, calibrated against one of the sets of standards, averaged or measured using new techniques.

Other standards

Another example of averaging in cytometry is *S. pseudocapsicum*. The DNA content for *S. pseudocapsicum* (2.59 pg) was measured by Temsch et al. (2010). In this work, *S. pseudocapsicum* was calibrated using standards of *Z. mays* 'CE-777' (5.18 pg), *H. vulgare* 'Ditta' (9.66 pg), *P. sativum* 'Kleine Rheinländerin' (8.84 pg) and *R. sativus* 'Saxa' (1.06 pg) (Vilhar et al., 2001). As can be seen, these values are very different from the classical Doležel's values. Furthermore, these values are not given in

the Vilhar et al. (2001). Simple calculations show that these values are the average of three values obtained by image cytometry of fixed and unfixed samples, where *P. sativum* was the primary standard. For example, *H. vulgare* 'Ditta' (9.66 pg) is the average of three values: 10.18, 9.63 and 9.19 pg (Vilhar et al., 2001; Table 2, 3) and is significantly lower than the Doležel's value of 10.43 pg.

The DNA content of *S. pseudocapsicum* based on our measurements is close to the data of Temsch et al. (2010) (2.562 pg, r2 = 0.996) when using the standards in the values of Vilhar et al. (2001) with regression line. In practice, the values of *S. pseudocapsicum* (2.59 pg) are linear with *S. lycopersicum* and *R. sativum* in the Doležel's value (1.11 and 1.96 pg).

Our direct measurements with multiple standards showed for *S. pseudocapsicum* 2C = 2.822 pg in the HML-based and 2.386 pg in the *O. s.*-based value. In the article by Temsch et al. (2020), P. Šmarda's unpublished value of 2C = 2.414 pg based on the *O. sativa* genome is given for *S. pseudocapsicum*, which is close to our data. *S. pseudocapsicum* proved to be a good standard in this work, with minimal variation, absence of endopolyploidy and metabolites, and high yield of nuclei. It is also evergreen and more convenient than annual species.

Commonly used standard is *B. perennis*. According to Temsch et al. (2022), the DNA content is 3.38 pg, according to unpublished data by J. Suda, primary standard is unknown. In practice, at this value, *B. perennis* is linear with *R. sativus* at a value of 1.11 pg and *S. lycopersicum* at a value of 1.96 pg, apparently one of these standards was used as the primary standard, because in Leong-Skornicková et al. (2007) it was recalibrated upwards using *G. max*. In the work of Veselý et al. (2012), the recalibration was performed with *S. lycopersicum* (1.735 pg) and linearly with all small *O. s.*-based standards. In our practice, *B. perennis* has polymorphism between individuals and it is worth monitoring its linearity with other standards; there are also problems with isolating nuclei in Otto buffer and deterioration of histograms is observed when co-chopping with tomato. It is therefore worth selecting a separate clone that is linear with other standards.

Independently, we can highlight such a standard as *A. thaliana* ecotype Columbia with an accepted 2C value of 0.321 pg. *A. thaliana* is considered to be as close as possible to real values, since it was obtained by flow cytometry with the primary standard *Caenorhabditis elegans*, which contains a minimum number of repeats (Bennet et al., 2003). However, according to AGI (Arabidopsis Genome Initiative) sequencing data, the genome size is 2C = 250 Mbp (0.256 pg). According to new data obtained by the hybrid short-read and long-read sequencing method, the genome size is intermediate between the previous data (1C = 133.7 Mbp/2C = 0.273 pg (Wang et al., 2022).

Polymorphism in the genome size of *A. thaliana* was studied by Schmuths et al. (2004) using *R. sativus* as a standard of 1.38 pg (Doležel et al., 1998) and obtained a 2C value for *A. thaliana* of 0.412 pg. Meister et al. (2005) determined the ratio of *O. sativa* to *A. thaliana* to be 2.545. When converted from *O. sativa* (0.795 pg), *A. thaliana* is 0.312 pg. Our direct measurements with multiple standards showed higher 2C values of 0.366 pg in the HML-based and 0.304 pg in the *O. s.*-based. Šmarda et al. (2019) apparently obtained a value of 0.304 pg for the wild type in the *O. s.*-based value, which is identical to our data. In our practice, there are no differences in genome size of Columbia and wild type. In practice, *A. thaliana* with a value of 0.321 pg is linear only with *R. sativus* and *S. lycopersicum* with a value of 1.11 pg and 1.96 pg. Thus, comparing the genome size of *A. thaliana* and *O. sativa* by cytometry will still give different data than the sequencing data. Another disadvantage is the high level of endopolyploidy.

Among the large genome standards, the most commonly used are *H. vulgare*, *S. cereale* and, to a lesser extent, *C. commosum*. These standards are quite stable, easy to use and linear with other large genome Doležel's standards. *H. vulgare* (10.43 pg) and *S. cereale* (16.19 pg) measured by Doležel et al. (1998) as an average between laboratories using *P. sativum* as a standard showed minimal inter-laboratory variation. According to our data, these standards are linear with *P. sativum* (9.09 pg), *A. cepa* (34.89 pg), *V. faba* (26.9 pg), *P. crispum* (4.5 pg) and others. They are probably no different from other varieties. Our laboratory compared the barley varieties Ditta and Scarlet and did not find any differences. Similarly, *S. cereale* 'Dankovsky' and 'Tatyana' do not differ in DNA content. The tetraploid varieties 'Tetra' and 'Sibir' having exactly double the genome (32.44 pg).

C. commosum is measured relative to *P. sativum* (9.09 pg) and has a value of 2C = 24.14 pg, which is linear with Doležel's standards and is convenient because it is a perennial. All three standards were measured for the first time in the *O. s.*-based value (Tables 2, 3).

On the basis of the DNA content of *H. vulgare* (10.04 pg) and *P. sativum* (8.75 pg) from Baranyi, Greilhuber (1998) developed her standards and B. Zonneveld first measured *A. americana* (15.90 pg) and then all other standards relative to *A. americana* (Zonneveld, Van Iren, 2000; Zonneveld, 2021). According to our direct measurements, the HML-based value of *A. americana* is 16.518 pg and the *O. s.*-based value is 14.122 pg.

In general, the use of perennial plants is logical and more convenient (Skaptsov et al., 2016; Zonneveld, 2021). Thus, among the standards with a small genome, *F. benjamina* (1.07/0.888 pg) and *E. japonicus* (0.688/0.569 pg) can be used. Large genomes include the perennials *C. commosum* and *A. tuberosum*.

Among the standards of Marie and Brown (1993) we can highlight *P. hybrida*. *P. hybrida* (2.85 pg) measured on the basis of CRBC (2.33 pg). It is an interesting coincidence that the standards using CRBC (2.33 pg) are linear with a slight deviation with *R. sativus* (1.11 pg) and *S. lycopersicum* (1.96 pg). For example, the rather rarely used standard *Trifolium repens* with a 2C value of 2.07 pg (Arumuganathan, Earle, 1991) is also linear with radish and tomato with a slight deviation. According to our direct measurements, *T. repens* corresponds to 2.326 pg in the HML-based value and 1.95 pg in the *O. s.*-based value. *P. hybrida*, according to our direct measurements, showed a DNA content value of 3.086 in HML-based value and 2.598 pg in *O. s.*-based value. The DNA content is stable between varieties, but it is important to remember that triploid hybrids exist.

A. fistulosum with 2C value 23.3–23.5 pg also corresponds to the standards of Marie and Brown (1993) (Ricroch et al., 2005; Smirnov et al., 2017). It is linear with the values for *P. sativum* (8.37 pg), *A. cepa* (32.4 pg) and other standards of Marie and Brown (1993). It is lower than the values of Bennett and Doležel, but the ratios are similar. Our direct measurements showed that *A. fistulosum* has a DNA content of 25.004 pg in the HML-based value and 21.426 pg in the *O. s.*-based value.

Another commonly used standard is *P. crispum*. According to our direct measurements, the DNA content of parsley is 4.492 pg in the HML-based value, which is close to the values of 4.5 and 4.46 obtained previously (Yokoya et al., 2000; Obermayer et al., 2002; Skaptsov et al., 2016). However, Obermayer et al. (2002) used Gardener's Delight tomato (2.0 pg) for the measurement of parsley DNA content. According to our data and previously obtained data, *P. crispum* is linear with *P. sativum* with values of 4.5 pg and 9.09 pg as shown by Clarc et al. (2016) and Skaptsov et al. (2016). *P. crispum* is a good standard, strongly linear with *P. sativum*, because of no endopolyploidy, no metabolites, minimal polymorphism between varieties.

In general, due to the bottleneck effect, plant varieties of the same species should in most cases be close in genome size and less polymorphic than wild species when talking about variety polymorphism. Excluding aneuploids, polyploids and species with extra chromosomes. For example, we analyzed tomato varieties (Stupice, Roma, Red Robin, Gardener's Delight), parsley (Giant of Italy, Moss Curled 2, Champion Moss, and even Greenery from the store), onion (Stuttgarter Riesen, Alice, Ailsa Craig, Greenery from the store), peas (Ctirad, Kelvedon Miracle, Greenery from the store), beans (Inovec, Chernyye russkiye), and variations in DNA content between varieties did not exceed 3 %. However, it is worth considering that trisomics and aneuploids are known for *S. lycopersicum* varieties (Banks, 1984). Similar genome stability between *P. sativum* varieties was previously shown by Greilhuber and Ebert (1994), with a maximum deviation of about 5 % using densitometry. Later, Bennet et al. (2000) showed the absence of DNA content polymorphism between *A. cepa* varieties. Clarc et al. (2016) also showed that the *A. cepa* variety 'Ailsa Craig' was close to the Doležel's value of 34.89 pg when calibrated using *V. faba* and *P. sativum*.

Therefore, the primary standard and instrumental variations are the main reasons for differences in DNA content between laboratories. Therefore, before comparing and discussing results, it is important to be aware of which standards with which 2C values have been used. It is possible to obtain very accurate results between laboratories using the same buffer, the same standard with the same 2C value. In addition, it is worth considering both the polymorphism of the samples and the polymorphism of the standards and constantly monitoring the linearity.

According to our study, cytometry and sequencing data are not linearly related. For example, *Arabidopsis* cytometry data are higher than sequencing data. *P. sativum* DNA content based on cytometry is lower than expected data when recalculated using the human genome. *O. s.*-based values from Veselý et al. (2012) are currently the closest to real data, the use of small genome standards between each other in practice show very accurate results. The

use of *P. sativum* (8.018 pg), *V. faba* (23.796 pg) and *A. cepa* (30.745 pg) leads to small differences when using cytometry. However, this can be critical when studying the monoploid genome in polyploid taxa and the linearity of standards for which differences have been identified should also be continuously monitored. If only *P. sativum*, *V. faba* and *A. cepa* are used, there will be no variation as they are linearly related. Therefore, it is possible to use *O. s.*-based standards in their original values without changes, as the differences are closer to the cytometric error. When instrumental error and interlaboratory differences are taken into account, the data are comparable.

In a set of standards with classical Doležel's values, only the 2C value of *R. sativus* and *S. lycopersicum* need to be modified. However, since we often observed variations in DNA content and ambiguous behavior in different buffers for *R. sativus*, *G. max, S. lycopersicum*, *B. perennis* and *Z. mays*, they can be replaced by *P. crispum*, *S. pseudocapsicum*, *F. benjamina* and *E. japonicus*.

The comparability of cytometric and sequencing data remains to be investigated as sequencing data accumulate. For example, based on sequencing, the 2C value of tomato is 1.634–1.84 pg according to different data (Tomato Genome Consortium, 2012; Su et al., 2021), soybean – 1.975–2.096 pg (Shen et al., 2018; Xinxin et al., 2022), maize – 4.454–4.683 pg (Chin et al., 2017; Hu, Resendre Jr., 2022) and radish – 0.974–1.032 pg (Shirasawa et al., 2020; Xu et al., 2023). Therefore, a set of standards based on classical Doležel's values is still relevant. The question is whether cytometry takes something into account or whether the sequencing method does not. Therefore, it is worthwhile to specify the value of the standard for subsequent recalculation in articles. Collecting data on the linearity of DNA content data between standards from different laboratories is very important to identify the most linear, trouble-free and low polymorphic under different conditions, buffers and regions to ensure comparability of data between laboratories.

Technical notes and observations

The standard for accuracy is the established and often quoted value of 3 % for both histogram peak variation (CV) and inter-measurement variation. It can be assumed that 3 % is the established error value of the cytometry method (Doležel, Bartos 2005; Doležel et al., 2007a; Sliwinska et al., 2021; Temsch, et al., 2021; Loureiro et al., 2023). This is largely due to the hardware error and linearity coefficient of the cytometer. In addition, there may be variations in the DNA content of the plant during the day and on different days, and in the quality and type of sample. Variations within 3 % are used to calculate the average 2C value. However, variations of 3 to 5 % are allowed, especially for plant groups with difficult sample preparation, desiccated plants and plants containing metabolites. Measurements above 3–5 % may indicate intraspecific polymorphism or changes in chromosome number or poor sample quality. This is especially true for small genomes where the size of a single chromosome is less than 3 % of the total DNA, especially for aneuploidy screening. In this case, validation by direct chromosome counting is desirable. There are several ways of calculating the average DNA content. Typically, at least three to five plants are sufficient to calculate the mean 2C, SD and CV (Burge et al., 2018). A more accurate approach is to examine at least three plants on three different days (9 measurements) when calculating the mean 2C value for a species (Doležel et al., 2007a). In population studies, the number of measurements typically ranges from three plants per population to several dozen or even hundreds of plants (especially if the intra-specific and intra-population variation are more than 3–5 %).

Characteristics labelled -area, -height and -width can be confusing to beginning researchers. For example, ECD-a, ECD-h, FSC-w, etc. Analyses can be performed in all three characteristics and the data will be different. These characteristics refer to the pulse of fluorescence and characterize the pulse in terms of width (signal duration), height (intensity) and area (total fluorescence) (pulse-w, pulse-h, pulse-a). In plant flow cytometry, it is common to use -area to measure DNA content because this characteristic is the mean fluorescence. For example, ECD-a, PI-a, DAPI-a, etc. It has also been shown that linearity is lost and CV increases when the pulse-h characteristic is used (Burge et al., 2018; Koutecký et al., 2023). Dot plot histograms of pulse-a vs. pulse-h (PI-a/PI-h) are useful for gating doublets and singlets, removing noise and destroyed nuclei, and improving the overall accuracy of the analysis. For example, doubling the DNA content of endopolyploids (2C, 4C, etc.) increases the area and height. In the case of doublets (superposition of nuclei in a flow cell), the area increases but the height remains unchanged. Doublets can also be separated by width (e. g. FSC-w), in which case the width of the doublets increases but the height remains the same. If a gating strategy with a different characteristic has

been used, this should be specified in the methods. The decision to use a particular characteristic depends on the cytometer. On some cytometers, linearity is often lost on the pulse-area characteristic, especially at high gain, probably due to the quality and dynamic range of the photomultiplier/photodiode. It is therefore useful to check linearity using standards or endopolyploid peaks. Examples of linearity of histogram peaks and calculations of DNA content in pulse-area and pulse-height are given in Supplement 2 on the journal's website.

To ensure the accuracy of the results, it is necessary to check the linearity of the standards and to follow the basic recommendations: use of live samples; use of internal standardization (sample and standard chopped together). External standardization (sample and standard chopped separately) is used for ploidy analysis or selection of standards, and the influence of metabolites can also be analyzed. Analysis of at least 1000 nuclei for each peak; avoidance of peak overlap, both 2C (G0/G1) and 4C (G2/M) and subsequent ones in the case of endopolyploidy; the difference between the 2C value of the standard and sample on modern cytometers is no more than 3 times without change in CV. As well as comparable peak heights of standard and sample, the difference in the number of nuclei is not more than 2–3 times. The CV is less than 3 %, for difficult objects not more than 5 %. The CV of the peak is particularly important as we have found that it is the CV that has the greatest influence on the difference. Therefore, if the nuclei are poorly isolated from the leaves, or the CV is high, it is necessary to try to isolate them from other organs, using seedlings or seeds.

If more than one standard is used, the publication should indicate which species was measured with which standard and at which value. This is necessary to recalculate the DNA content to a different value of the 2C standard. For example, it is very easy to recalculate data from HML-based to *O. s.*-based values if the same standard is used: new 2C value of the sample = (previous 2C value of the sample / previous 2C value of the standard) * new 2C value of the standard. When working with taxa with multiple ploidy levels and more than one standard is required, it is possible to calibrate a diploid sample for greater accuracy and use it as a standard in combination with the main standard (Šmarda et al., 2008; Sokoloff et al., 2024).

Of particular note are fixed samples (dried in silica gel or herbarium samples). Some studies indicate that dried samples not only give poor histograms with peak CVs of 5 % or more but also overestimate or underestimate results. Thus, only the approximate genome size and ploidy can be studied on fixed material (Bainard et al., 2011; Suda, Travnicek, 2014; Wang, Yang, 2016). In our practice, the 2C value of dried material is mostly too high, how much higher is very species specific. We observed a maximum overestimation of up to 15–20 %. Often, the higher the CV of the peak, the greater the deviation, so sometimes you can make a mistake when interpreting ploidy when working with tri-, hexaploids, etc.) It is possible to use a desiccated standard. This partially reduces the deviation but degrades the quality of the histograms. Furthermore, it is necessary to dry the standard on the same day as the specimen. And for most standards, the effect of drying has not been studied, i.e. whether the fluorescence will increase or decrease.

In addition, we observed a similar phenomenon when studying long-term stored seeds. The 2C value of the embryo is also overestimated by a maximum of 15–20 %. It is also possible, due to the destruction of DNA in the embryo nuclei and the preservation of the endosperm nuclei, to confuse the endosperm peak with the embryo peak. This is particularly the case in the Apiaceae family, which has a very small embryo. The embryo peak is often not visible on histograms and is poorly discernible even in fresh seeds due to the predominance of the endosperm peak.

However, if it is not possible to use live material due to the distance of the laboratory or the duration of the expedition, the effect of drying must be tested on the object of investigation, high quality dried silica gel should be used, CV peaks should remain at the same level or not exceed 3–5 %, the material should be analyzed within 3 months of drying, try to collect seeds for germination or a small amount of live material for comparison.

As pointed out by Bainard et al. (2011), the effects of drying on the histogram can be overcome by the use of gating. Indeed, gating removes excess noise and improves the quality of the histogram and CV peaks but does not have a significant effect on the result. In most cases, an increase in the 2C value appears to be associated with the disruption of protein-nucleic acid complexes and tightly packed DNA structures, as in the case of Otto buffer, under the influence of nucleases, drying or other factors, thereby increasing the fluorescence of the dye/DNA complex.

The effect of desiccation is very species-specific; ferns and many spore-bearing plants

tolerate desiccation well. For example, nuclei are well preserved in herbarium samples of the genus *Selaginella* that have been stored for up to 50 years. It has even been possible to isolate nuclei from an almost century-old herbarium. (Skaptsov et al., 2020). The effect of drying should be tested against live samples. CV of peaks and DNA content should not vary.

A very low 2C peak can also occur in endopolyploid samples and a common mistake is that it appears behind a threshold. For example, in *Arabidopsis* or Orchidaceae, in different parts and organs of the plant, the 2C peak may not be visible due to noise or may be completely absent, but the nuclei of the 2C peak can be observed on dot-plot histograms. Therefore, a 4C peak may be mistaken for a 2C peak, leading to misinterpretation of DNA content and ploidy. It is therefore necessary to ensure that there are no events on the channel with two times less fluorescence to the left of the first peak. However, it should be remembered that there is a rare type of endopolyploidy – Progressive Partial Endoreplication (PPE), which is typical to Orchidaceae. In PPE, the ratio of peaks to each other can be significantly different from the standard ratios. The 2C peak may be absent even in leaves. Therefore, different organs, seeds, meristematic tissues, embryos or generative parts must be examined, as the unequal ratio between peaks and the frequent absence of the 2C peak can lead to measurement errors and misinterpretation of DNA content and ploidy (Trávníček et al., 2015; Brown et al., 2017).

Both the polymorphism of the samples and the polymorphism of the standards contribute to the difference in measurements between different laboratories. For example, if a sample is measured with the same standard according to all rules, the error is 1–1.5 %, and with different standards, even linear to each other, the error is 2–5 %. In a pairwise comparison, this was found for radish, soya bean, bellis and, to a lesser extent, tomato. For example, the difference between leaves of different ages in soybean (from 2.4–2.6 pg) and tomato (2.03–2.11) relative to pea, especially in very young apical light green leaves, may also be due to DNA conformation. And of course, the quality of the sample and standard itself, the content of metabolites, is important, and as noted by Bainard et al. (2011) even the time of collection (spring – autumn).

Bainard et al. (2011) found that the maximum influence on the results was related to the instrument. And he admitted that the maximum deviation for desiccated material could reach 10 %, which is comparable to the polymorphism between laboratories. In our work, the difference between CyFlow PA and Cytoflex was not significant when the conditions were met (CV, counts, and especially for CyFlow PA, the standard/sample difference was not more than 2-fold). The effect of buffer in the work of Bainard et al. (2011) received less attention because of the use of only $MgSO_4$ and LB01 buffers (one-step procedure) without the use of Otto buffers.

The influence of the operator on the results is often discussed. The influence of the operator seems to be the manner of chopping; crushing should be sandwich-like, fast and fine with short cutting movements; if the sample is crushed for a long time, the blade becomes blunt and begins to squash the nuclei.

The popularity of Otto and Tris-$MgCl_2$ buffers is due to the fact that they are the main buffers in the commercial Partec nuclear isolation kits (Phosser et al., 1995; Greilhuber et al., 2007). At the same gain in Otto, the fluorescence differs to a greater extent, apparently due to changes in the composition of histones and secondary structures. For example, removal of histones by hydrochloric acid leads to a twofold increase in DAPI fluorescence (Darzynkiewicz et al., 1984). Fluorescence in LB01 buffer is less than in Otto but more than in Tris-$MgCl_2$ buffer, apparently due to the presence of the chromatin stabilizer spermine in the buffer. This has an effect on the structure of the chromatin and the binding of the dye to the nucleic acids. As for other stabilizers, cations and polyamines, their effectiveness decreases in the following order: spermine > spermidine > Mg2+ > Na+ > Tris-HCl buffer alone at pH 7.3 (Hou et al., 2001).

Protocols and recipes for solutions and an example of the requirements for histograms are given in Supplement 2 on the journal's website.

Acknowledgements

We are grateful to the M. N. Lomonosova and T. V. Pankova (Central Siberian Botanical Garden of the Siberian Branch of the RAS) for providing seeds of Doležel's standards and E. L. Gorbyleva (Siberian Institute of Plant Physiology and Biochemistry of the Siberian Branch of the RAS) for providing seeds of *A. thaliana* ecotype Columbia. This work was supported by the State Assignment of the Altai State University (project FZMW-2023-0008).

REFERENCES / ЛИТЕРАТУРА

Arumuganathan K., Earle E. D. 1991. Nuclear DNA content of some important plant species. *Pl. Molec. Biol. Rep.* 9: 208–218. DOI: 10.1007/BF02672069

Bainard J. D., Husband B. C., Baldwin S. J., Fazekas A. J., Gregory T. R., Newmaster S. G., Kron P. 2011. The effects of rapid desiccation on estimates of plant genome size. *Chromosome Res.* 19(6): 825–842. DOI: 10.1007/s10577-011-9232-5

Banks P. 1984. A new diploid chromosome number for tomato (*Lycopersicon esculentum*)? *Can. J. Genet. Cytol.* 26(5): 636–639. DOI: 10.1139/g84-099

Baranyi M., Greilhuber J. 1996. Flow cytometric and Feulgen densitometric analysis of genome size variation in Pisum. *Theor. Appl. Genet*. 92: 297–307. DOI: 10.1007/BF00223672

Bennett M. D. 1972. Nuclear DNA content and minimum generation time in herbaceous plants. *Proc. R. Soc. Lond. B Biol. Sci.* 181 (1063): 109–135. DOI: 10.1098/rspb.1972.0042

Bennett M. D., Bhandol P., Leitch I. J. 2000. Nuclear DNA amounts in angiosperms and their modern uses – 807 New Estimates. *Ann. Bot*. 86(4): 859–909. DOI:10.1006/anbo.2000.1253

Bennett M., Johnston J., Hodnett G., Price H. 2000. *Allium cepa* L. cultivars from four continents compared by flow cytometry show nuclear DNA constancy. *Ann. Bot*. 85(3): 351–357. DOI: 10.1006/anbo.1999.1071

Bennett M. D., Leitch I. J. 1995. Nuclear DNA amounts in angiosperms. *Ann. Bot*. 76(2): 113–176. DOI: 10.1006/anbo.1995.1085

Bennett M. D., Leitch I. J. 1997. Nuclear DNA amounts in angiosperms – 583 new estimates. *Ann. Bot.* 80(2): 169–196. DOI: 10.1006/anbo.1997.0415

Bennett M. D., Leitch I. J. 2001. Nuclear DNA amounts in pteridophytes. *Ann. Bot.* 87(3): 335–345. DOI:10.1006/anbo.2000.1339

Bennett M. D., Leitch I. J. 2005. Nuclear DNA amounts in angiosperms: progress, problems and prospects. *Ann. Bot.* 95(1): 45–90. DOI: 10.1093/aob/mci003

Bennett M. D., Leitch I. J. 2011. Nuclear DNA amounts in angiosperms: targets, trends and tomorrow. *Ann. Bot*. 107(3): 467–590. DOI: 10.1093/aob/mcq258

Bennett M. D., Leitch I. J., Price H. J., Johnston J. S. 2003. Comparisons with *Caenorhabditis* (100 Mb) and *Drosophila* (175 Mb) using flow cytometry show genome size in *Arabidopsis* to be 157 Mb and thus 25 % larger than the Arabidopsis genome Initiative estimate of 125 Mb. *Ann. Bot.* 91(5): 547–557. DOI: 10.1093/aob/mcg057

Bennett M. D., Smith J. B. 1976. Nuclear DNA amounts in angiosperms. *Philos Trans R. Soc. Lond. B Biol. Sci.* 274 (933): 227–274. DOI: 10.1098/rstb.1976.0044. PMID: 6977

Bennett M. D., Smith J. B., Heslop-Harrison J. S. 1982. Nuclear DNA amounts in angiosperms. *Proc. R. Soc. Lond. B Biol. Sci.* 216: 179–199. DOI: 10.1098/rspb.1982.0069

Bourge M., Brown S., Siljak-Yakovlev S. 2018. Flow cytometry as tool in plant sciences, with emphasis on genome size and ploidy level assessment. *Genetics and Applications*. 2, 2: 1–12. DOI: 10.31383/ga.vol2iss2pp1-12

Brown S. C., Bourge M., Maunoury N., Wong M., Wolfe Bianchi M., Lepers-Andrzejewski S., Besse P., Siljak-Yakovlev S., Dron M., Satiat-Jeunemaître B. 2017. DNA Remodeling by strict partial endoreplication in Orchids, an original process in the plant kingdom. *Genome Biol. Evol*. 9(4): 1051–1071. DOI: 10.1093/gbe/evx063

Chen J., Wang Z., Tan K., Huang W., Shi J., Li T., Hu J., Wang K., Wang C., Xin B., Zhao H., Song W., Hufford M. B., Schnable J. C., Jin W., Lai J. 2023. A complete telomere-to-telomere assembly of the maize genome. *Nat. Genet.* 55: 1221–1231. DOI: 10.1038/s41588-023-01419-6

Clark J., Hidalgo O., Pellicer J., Liu H., Marquardt J., Robert Y., Christenhusz M., Zhang S., Gibby M., Leitch I. J., Schneider H. 2016. Genome evolution of ferns: evidence for relative stasis of genome size across the fern phylogeny. *New Phytol*. 210(3): 1072–1082. DOI: 10.1111/nph.13833

Darzynkiewicz Z., Traganos F., Kapuscinski J., Staiano-Coico L., Melamed M. R. 1984. Accessibility of DNA in situ to various fluorochromes: relationship to chromatin changes during erythroid differentiation of Friend leukemia cells. *Cytometry A* 5(4): 355–363. DOI: 10.1002/cyto.990050411

Doležel J., Bartos J. 2005. Plant DNA flow cytometry and estimation of nuclear genome size. *Ann. Bot*. 95(1): 99–110. DOI: 10.1093/aob/mci005

Doležel J., Bartoš J., Voglmayr H., Greilhuber J. 2003. Nuclear DNA content and genome size of trout and human. *Cytometry A* 51: 127–128. DOI: 10.1002/cyto.a.10013

Doležel J., Doleželová M., Novák F. 1994. Flow cytometric estimation of nuclear DNA amount in diploid bananas (*Musa acuminata* and *M. balbisiana*). *Biol. Plant*. 36: 351–357. DOI: 10.1007/BF02920930

Doležel J., Greilhuber J. 2010. Nuclear genome size: are we getting closer? *Cytometry A* 19: 103–106. DOI: 10.1002/cyto.a.20915

Doležel J., Greilhuber J., Lucretti S., Meister A., Lysák M., Nardi L., Obermayer R. 1998. Plant genome size estimation by flow cytometry: inter-laboratory comparison. *Ann. Bot*. 82: 17–26. DOI: 10.1093/oxfordjournals.aob.a010312

Doležel J., Greilhuber J., Suda J. 2007a. Estimation of nuclear DNA content in plants using flow cytometry. *Nat. Prot.* 2(9): 2233–2244. DOI: 10.1038/nprot.2007.310

Doležel J., Greilhuber J., Suda J. 2007b. Flow cytometry with plants: an Overview. In: J. Doležel, J. Greilhuber, J. Suda (eds.). *Flow cytometry with plant cells*. Weinheim: Wiley-VCH. Pp. 41–66. DOI: 10.1002/9783527610921.ch3

Doležel J., Sgorbati S., Lucretti S. 1992. Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. *Phys. Plant*. 85: 625–631. DOI: 10.1111/j.1399-3054.1992.tb04764.x

Galbraith D. W., Harkins K. R., Maddox J. M., Ayres N. M., Sharma D. P., Firoozabady E. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* 220(4601): 1049–1051. DOI: 10.1126/science.220.4601.1049

Greilhuber J., Doležel J., Lysák MA., Bennett MD. 2005. The origin, evolution and proposed stabilization of the terms 'genome size' and 'C-value' to describe nuclear DNA contents. *Ann. Bot*. 95(1): 255–260. DOI: 10.1093/aob/mci019

Greilhuber J., Ebert I. 1994. Genome size variation in *Pisum sativum*. *Genome* 37: 646–655. DOI: 10.1139/g94-092

Greilhuber J., Temsch E. M., Loureiro J. C. M. 2007. Nuclear DNA content measurement. In: J. Doležel, J. Greilhuber, J. Suda (eds.). *Flow cytometry with plant cells*. Weinheim: Wiley-VCH. Pp. 67–101. DOI: 10.1002/9783527610921.ch4

Heller F. O. 1973. DNS-Bestimmung an Keimwurzeln von *Vicia faba* L. mit Hilfe der Impulseytophotometcie. *Ber. Deutsch. Bot. Gesellschaft.* 86, 437–441. DOI: 10.1111/j.1438-8677.1973.tb02427.x

Hornych O., Ekrt L., Riedel F., Koutecký P., Košnar J. 2019. Asymmetric hybridization in central European populations of the *Dryopteris carthusiana* group. *Am. J. Bot*. 106: 1477–1486. DOI: 10.1002/ajb2.1369

Hou M. H., Lin S. B., Yuann J. M., Lin W. C., Wang A. H., Kan Ls. L. 2001. Effects of polyamines on the thermal stability and formation kinetics of DNA duplexes with abnormal structure. *Nucleic Acids Res*. 29(24): 5121–5128. DOI: 10.1093/nar/29.24.5121

Hu Y., Resende Jr. M. F. R. 2022. Maize genome assembly with PacBio reads. *Bio-101*: e4456. DOI: 10.21769/BioProtoc.4456

International Rice Genome Sequencing Project. 2005. The map-based sequence of the rice genome. *Nature* 436: 793–800. DOI: 10.1038/nature03895

Johnston J. S., Bennett M. D., Rayburn A. L., Galbraith D.W., Price H. J. 1999. Reference standards for determination of DNA content of plant nuclei. *American Journal of Botany.* 86(5): 609–613. DOI: 10.2307/2656569

Koutecký P., Smith T., Loureiro J., Kron P. 2023. Best practices for instrument settings and raw data analysis in plant flow cytometry. *Cytometry A* 103(12): 953–966. DOI: 10.1002/cyto.a.24798

Kron P., Husband B. C. 2012. Using flow cytometry to estimate pollen DNA content: improved methodology and applications. *Ann. Bot*. 110(5): 1067–1078. DOI: 10.1093/aob/mcs167

Kubešová M., Moravcová L., Suda J., Jarošík V., Pyšek P. 2010. Naturalized plants have smaller genomes than their non-invading relatives: a flow cytometric analysis of the Czech alien flora. *Preslia* 82: 81–96.

Leong-Skornicková J., Sída O., Jarolímová V., Sabu M., Fér T., Trávnícek P., Suda J. 2007. Chromosome numbers and genome size variation in Indian species of *Curcuma* (Zingiberaceae). *Ann. Bot*. 100(3): 505–526. DOI: 10.1093/aob/mcm144

Loureiro J., Čertner M., Lučanová M., Sliwinska E., Kolář F., Doležel J., Garcia S., Castro S., Galbraith D. W. 2023. The use of flow cytometry for estimating genome sizes and DNA ploidy levels in plants. *Methods Mol. Biol*. 2672: 25–64. DOI: 10.1007/978-1-0716-3226-0_2

Loureiro J., Rodriguez E., Doležel J., Santos C. 2006. Comparison of four nuclear isolation buffers for plant DNA flow cytometry. *Ann. Bot*. 98(3): 679–689. DOI: 10.1093/aob/mcl141

Loureiro J., Rodriguez E., Doležel J., Santos C. 2007. Two new nuclear isolation buffers for plant DNA flow cytometry: a test with 37 species. *Ann. Bot*. 100(4): 875–888. DOI: 10.1093/aob/mcm152

Lysák M. A., Doležel J. 1998. Estimation of nuclear DNA content in *Sesleria* (Poaceae). *Caryologia* 51: 123–132. DOI: 10.1080/00087114.1998.10589127

Marie D., Brown S. C. 1993. A cytometric exercise in plant DNA histograms, with 2C-values for 70 species. *Biol. Cell.* 78: 41–51. DOI: 10.1016/0248-4900(93)90113-S

Meister A. 2005. Calculation of binding length of base-specific DNA dyes by comparison of sequence and flow cytometric data. Application to *Oryza sativa* and *Arabidopsis thaliana*. *J. Theor. Biol.* 232: 93–97. DOI: 10.1016/j.jtbi.2004.07.022

Nurk S., Koren S., Rhie A., Rautiainen M., Bzikadze A. V., Mikheenko A. et al. 2022. The complete sequence of a human genome. *Science* 376(6588): 44–53. DOI: 10.1126/science.abj6987

Obermayer R., Leitch I. J., Hanson L., Bennett M. D. 2002. Nuclear DNA C-values in 30 species double the familial representation in pteridophytes. *Ann. Bot*. 90(2): 209–217. DOI: 10.1093/aob/mcf167

Otto F. 1990. DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. *Methods Cell Biol.* 33: 105–110. DOI: 10.1016/s0091-679x(08)60516-6

Pfosser M., Amon A., Lelley T., Heberle-Bors E. 1995. Evaluation of sensitivity of flow cytometry in detecting aneuploidy in wheat using disomic and ditelosomic wheat-rye addition lines. *Cytometry A* 21(4): 387–393. DOI: 10.1002/cyto.990210412

Praça-Fontes M. M., Carvalho C. R., Clarindo W. R., Cruz C. D. 2011. Revisiting the DNA C-values of the genome size-standards used in plant flow cytometry to choose the "best primary standards". *Plant Cell Rep*. 30(7): 1183–1191. DOI: 10.1007/s00299-011-1026-x

Ricroch A., Yockteng R., Brown S. C., Nadot S. 2005. Evolution of genome size across some cultivated *Allium* species. *Genome* 48: 511-520. DOI: 10.1139/g05-017

Rosado T. B., Clarindo W. R., Carvalho C. R. 2009. An integrated cytogenetic, flow and image cytometry procedure used to measure the DNA content of *Zea mays* A and B chromosomes. *Plant Sci*. 176(1): 154–158. DOI: 10.1016/j.plantsci.2008.10.007

Rosato M., Chiavarino A., Naranjo C., Hernandez J., Poggio L. 1998. Genome size and numerical polymorphism for the B chromosome in races of maize (*Zea mays* ssp. *mays*, Poaceae). *A. J. Bot*. 85(2): 168–174. DOI: 10.2307/2446305

Schmuths H., Meister A., Horres R., Bachmann K. 2004. Genome size variation among accessions of *Arabidopsis thaliana*. *Ann. Bot.* 93(3): 317–321. DOI: 10.1093/aob/mch037

Shang L., He W., Wang T., Yang Y., Xu Q., Zhao X., Yang L., Zhang H., Li X., Lv Y., Chen W., Cao S., Wang X., Zhang B., Liu X., Yu X., He H., Wei H., Leng Y., Shi C., Guo M., Zhang Z., Zhang B., Yuan Q., Qian H., Cao X., Cui Y., Zhang Q., Dai X., Liu C., Guo L., Zhou Y., Zheng X., Ruan J., Cheng Z., Pan W., Qian Q. 2023. A complete assembly of the rice Nipponbare reference genome. *Mol. Plant* 16(8): 1232–1236. DOI: 10.1016/j.molp.2023.08.003

Shen Y., Liu J., Geng H., Zhang J., Liu Y., Zhang H., Xing S., Du J., Ma S., Tian Z. 2018. De novo assembly of a Chinese soybean genome. *Sci. China Life Sci*. 61(8): 871–884. DOI: 10.1007/s11427-018-9360-0

Shirasawa K., Hirakawa H., Fukino N., Kitashiba H., Isobe S. 2020. Genome sequence and analysis of a Japanese radish (*Raphanus sativus*) cultivar named 'Sakurajima Daikon' possessing giant root. *DNA Res*. 27, 2: dsaa010. DOI: 10.1093/dnares/dsaa010

Skaptsov M. V., Smirnov S. V., Kutsev M. G., Shmakov A. I. 2016. Problems of a standardization in plant flow cytometry. *Turczaninowia* 19, 3: 120–122. [In Russian] (***Скапцов М. В., Смирнов С. В., Куцев М. Г., Шмаков А. И.*** Проблемы стандартизации в проточной цитометрии растений // Turczaninowia, 2016. Т. 19, № 3. С. 120–122). DOI: 10.14258/turczaninowia.19.3.9

Skaptsov M. V., Vaganov A. V., Kechaykin A. A., Kutsev M. G., Smirnov S. V., Dorofeev V. I., Borodina-Grabovskaya A. E., Seregin A. P., Sinitsina T. A., Friesen N., Zhang X., Shmakov A. I. 2020. The cytotypes variability of the complex *Selaginella sanguinolenta* s. l. *Turczaninowia*. 23, 2: 5–14. DOI: 10.14258/turczaninowia.23.2.1

Sliwinska E., Loureiro J., Leitch I. J., Šmarda P., Bainard J., Bureš P., Chumová Z., Horová L., Koutecký P., Lučanová M., Trávníček P., Galbraith D. W. 2022. Application-based guidelines for best practices in plant flow cytometry. *Cytometry A* 101(9): 749–781. DOI: 10.1002/cyto.a.24499

Šmarda P., Bureš P., Horová L., Foggi B., Rossi G. 2008. Genome size and gc content evolution of *Festuca*: ancestral expansion and subsequent reduction. *Ann. Bot*. 101(3): 421–433. DOI:10.1093/aob/mcm307

Šmarda P., Bureš P., Horová L., Leitch I. J., Mucina L., Pacini E., Tichý L., Grulich V., Rotreklová O. 2014. Ecological and evolutionary significance of genomic GC content diversity in monocots. *Proc. Natl. Acad. Sci. U.S.A.* 111(39): 4096–4102. DOI: 10.1073/pnas.1321152111

Šmarda P., Knápek O., Šilerová A., Horová L., Grulich V., Danihelka J., Veselý P., Šmerda J., Rotreklová O., Bureš P. 2019. Genome sizes and genomic guanine+cytosine (GC) contents of the Czech vascular flora with new estimates for 1700 species. *Preslia* 91(2): 117–142. DOI: 10.23855/preslia.2019.117. 2019

Smirnov S., Skaptsov M., Shmakov A., Fritsch R., Friesen N. 2017. Spontaneous hybridization among *Allium tulipifolium* and *A. robustum* (*Allium* subg. *Melanocrommyum*, Amaryllidaceae) under cultivation. *Phytotaxa* 303: 155. DOI: 10.11646/phytotaxa.303.2.5

Sokoloff D. D., Degtjareva G. V., Skaptsov M. V., Vislobokov N. A., Kirejtshuk A. G., Sennikov A. N. et al. 2024. Diploids and tetraploids of *Acorus* (Acoraceae) in temperate Asia are pseudocryptic species with clear differences in micromorphology, DNA sequences and distribution patterns, but shared pollination biology. *Taxon* 73: 718–761. DOI: 10.1002/tax.13173

Su X., Wang B., Geng X., Du Y., Yang Q., Liang B., Meng G., Gao Q., Yang W., Zhu Y., Lin T. 2021. A high-continuity and annotated tomato reference genome. *BMC Genomics* 22, 1: 898. DOI: 10.1186/s12864-021-08212-x

Suda J., Krahulcová A., Trávnícek P., Rosenbaumová R., Peckert T., Krahulec F. 2007. Genome size variation and species relationships in *Hieracium* sub-genus *Pilosella* (Asteraceae) as inferred by flow cytometry. *Ann. Bot*. 100(6): 1323–1335. DOI: 10.1093/aob/mcm218

Suda J., Leitch I. J. 2010. The quest for suitable reference standards in genome size research. *Cytometry A* 77(8): 717–720. DOI: 10.1002/cyto.a.20907. PMID: 20653010

Suda J., Trávnícek P. 2006. Reliable DNA ploidy determination in dehydrated tissues of vascular plants by DAPI flow cytometry – new prospects for plant research. *Cytometry A* 69(4): 273–280. DOI: 10.1002/cyto.a.20253. PMID: 16528735.

Suda J. 2009. *An employment of flow cytometry into plant biosystematics.* PhD thesis. Charles University in Prague. 50 pp.

Temsch E. M., Greilhuber J., Krisai R. 2010. Genome size in liverworts. *Preslia* 82: 63–80.

Temsch E. M., Koutecký P., Urfus T., Šmarda P., Doležel J. 2022. Reference standards for flow cytometric estimation of absolute nuclear DNA content in plants. *Cytometry A* 101(9): 710–724. DOI: 10.1002/cyto.a.24495

The Arabidopsis Genome Initiative. 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana. Nature* 408: 796–815. DOI: 10.1038/35048692

Tiersch T. R., Chandler R. W., Wachtel S. S., Elias S. 1989. Reference standards for flow cytometry and application in comparative studies of nuclear DNA content. *Cytometry A* 10(6): 706–10. DOI: 10.1002/cyto.990100606

Tomato Genome Consortium. 2012. The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* 485, 7400: 635–641. DOI: 10.1038/nature11119

Trávníček P., Ponert J., Urfus T., Jersáková J., Vrána J., Hřibová E., Doležel J., Suda J. 2015. Challenges of flow-cytometric estimation of nuclear genome size in orchids, a plant group with both whole-genome and progressively partial endoreplication. *Cytometry A* 87(10): 958 – 966. DOI: 10.1002/cyto.a.22681

Van't Hof J. 1965. Relationships between mitotic cycle duration S period duration and average rate of DNA synthesis in root meristem cells of several plants. *Exp. Cell Res.* 39: 48–58. DOI: 10.1016/0014-4827(65)90006-6

Veleba A., Šmarda P., Zedek F., Horová L., Šmerda J., Bureš P. 2017. Evolution of genome size and genomic GC content in carnivorous holokinetics (Droseraceae). *Ann. Bot.* 119(3): 409–416. DOI: 10.1093/aob/mcw229

Veselý P., Bures P., Smarda P., Pavlícek T. 2012. Genome size and DNA base composition of geophytes: the mirror of phenology and ecology? *Ann. Bot*. 109(1): 65–75. DOI: 10.1093/aob/mcr267

Vilhar B., Greilhuber J., Dolenc-Koce J., Temsch E. M., Dermastia M. 2001. Plant genome size measurement with DNA image cytometry. *Ann. Bot.* 87(6): 719–728.

Wang G., Yang Y. 2016. The effects of fresh and rapid desiccated tissue on estimates of Ophiopogoneae genome size. *Plant Divers*. 38(4): 190–193. DOI: 10.1016/j.pld.2016.08.001

Xu L., Wang Y., Dong J., Zhang W., Tang M., Zhang W., Wang K., Chen Y., Zhang X., He Q., Zhang X., Wang K., Wang L., Ma Y., Xia K., Liu L. 2023. A chromosome-level genome assembly of radish (*Raphanus sativus* L.) reveals insights into genome adaptation and differential bolting regulation. *Plant Biotechnol. J*. 21(5): 990–1004. DOI: 10.1111/pbi.14011

Yi X., Liu J., Chen S., Wu H., Liu M., Xu Q., Lei L., Lee S., Zhang B., Kudrna D., Fan W., Wing RA., Wang X., Zhang M., Zhang J., Yang C., Chen N. 2022. Genome assembly of the JD17 soybean provides a new reference genome for comparative genomics. *G3 (Bethesda)*. 12, 4: jkac017. DOI: 10.1093/g3journal/jkac017

Yokoya K., Roberts A. V., Mottley J., Lewis R., Brandham P. E. 2000. Nuclear DNA amounts in roses. *Ann. Bot*. 85(4): 557–561. DOI: 10.1006/anbo.1999.1102

Zonneveld B. J. M. 2021. Selected perennial plants do provide convenient standards for the determination of genome sizes with flow cytometry. *Plant Syst. Evol*. 307: 28. DOI: 10.1007/s00606-021-01747-2

Zonneveld B. J. M., Van Iren F. 2000. Flow cytometric analysis of DNA content in *Hosta* reveals ploidy chimeras. *Euphytica* 111: 105–110. DOI: 10.1023/A:1003879408413