Botanica Pacifica. A journal of plant science and conservation. 2018. 7(1): 89–92
DOI: 10.17581/bp.2018.07113

Flow cytometry analysis of the relative content of nuclear DNA in *Nitraria schoberi* L. seeds

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Manuscript received: 20.03.2018
Review completed: 05.05.2018
Accepted for publication: 18.05.2018
Published online: 19.05.2018

ABSTRACT

The effectiveness of using the flow cytometry method (FCM) to analyze the genome size of the genus *Nitraria* L. plants is discussed for the first time, a technique having been developed to study the relative nuclear DNA content in resting seeds of *Nitraria schoberi* L. The effect of adding polyvinylpyrrolidone antioxidant to the extracting buffer was studied to assess the presence of metabolites inhibiting nuclei staining with propidium iodide in leaves of a standard sample *Raphanus sativus* L. and *N. schoberi* seeds. The existence of inhibitors in leaves of the standard *Raphanus sativus* L. 'Saxa' was established. Intensity of fluorescence of nuclei isolated from *N. schoberi* seeds did not change under the antioxidant, indicating the absence of the staining inhibitor in the seeds. In addition, *N. schoberi* seeds are a convenient material for analysis of genome size, as they do not contain endosperm, are easily transported and easily stored.

Keywords: *Nitraria schoberi*, flow cytometry, genome size, relative DNA content

РЕЗЮМЕ

Банаев Е.В., Томошевич М.А., Воронкова М.С. Особенности анализа относительного содержания ядерной ДНК в семенах *Nitraria schoberi* L. методом проточной цитометрии. Впервые проведена оценка эффективности использования метода проточной цитометрии (FCM) для анализа размера генома растений рода *Nitraria* L., разработана методика исследования относительного содержания ядерной ДНК в покоящихся семенах *Nitraria schoberi* L. Для оценки присутствия метаболитов, ингибирующих окрашивание ядер иодидом пропидия, в листьях стандартного образца *Raphanus sativus* L. 'Saxa', и семенах *N. schoberi* проведено изучение влияния добавления антиоксиданта поливинилпирролидона в экстрагирующий буфер. Установлено наличие ингибиторов в листьях стандартного образца *Raphanus sativus* L. 'Saxa'. Интенсивность флюоресценции ядер, выделенных из семян *N. schoberi*, не меняется под действием антиоксиданта, что свидетельствует об отсутствии в семенах ингибиторов окрашивания. Кроме того, семена *N. schoberi* являются удобным материалом для анализа размера генома, поскольку не содержат эндосперм, легко транспортируются и хорошо хранятся.

Ключевые слова: *Nitraria schoberi*, проточная цитометрия, размер генома, относительное содержание ДНК

Plant cell nuclei contain hereditary information, which is why studies of nuclear DNA are of fundamental importance in solving complex biological questions. An effective method for estimating genome size is flow cytometry (FCM), successfully used in recent decades for assessing the genetic diversity of various plant organisms (Galbraith 2010); for solving taxonomic issues (Doležel et al. 2007, Loureiro et al. 2008, Rayburn et al 2009), questions regarding species evolution and dynamics (Bennett et al. 2005, Lee 2002, Loureir et al. 2010, Bennert et al. 2011), problems of natural hybridization (Price 1988, Kur et al. 2012, Winter et al. 2013, Hanusova et al. 2014), and polyploidy (Pecinka et al. 2006).

Plants of the genus *Nitraria* L. are typical halophytes, usually confined to intrazonal communities. In the view of Komarov (1908), the phylogenetic origin of this genus predates the formation of the desert belt along Gondwanan seacoasts in the Old World and Australia, and the slight morphological differentiation of species to the present day does not allow us to draw conclusions about their genesis. Populations of many *Nitraria* species are largely isolated from each other, which makes this genus a unique model for studying genetic differentiation.

The goal of this study was to develop a methodology for determining the genome size in seeds of *Nitraria* plants using FCM.

To estimate the genome size of *N. schoberi*, seeds were harvested on the shore of Lake Bagan in West Siberia (53°53'54.54"N, 77°8'33.60"E), the natural habitat of *Nitraria*. As an internal standard we used fresh leaves of *Raphanus sativus* L. 'Saxa[32]' (2C DNA content = 1.11 pg) grown from seeds obtained from the Laboratory of Molecular Cytogenetics and Cytometry of the Institute of Experimental Bo-

tany of the Academy of Sciences of the Czech Republic, in Olomouc-Holice (Doležel et al. 1992).

All flow-cytometric measurements were performed in the Central Siberian Botanical Garden of the Siberian Branch of the Russian Academy of Sciences (Novosibirsk). The analysis was carried out using a Cy Flow Space cytometer (manufactured by Sysmex Partec, Germany) with a laser radiation source of 532 nm. The plant DNA content was determined by flow cytometry, staining isolated nuclei with propidium iodide (PI).

A *N. schoberi* germ extracted from a seed was ground using a sharp blade together with an appropriate amount of the internal standard (*Raphanus sativus* L. 'Saxa[32]') in 500 µl of a cooled extracting buffer (Nuclei Extraction Buffer) (Sysmex Partec) according to the manufacturer's protocol in Petri plastic cups. Samples were incubated at room temperature for two minutes.

The effect of adding polyvinylpyrrolidone (PVP) (Mw ~29000) (Sigma-Aldrich, USA) antioxidant to the extracting buffer was studied to assess the presence of metabolites inhibiting nuclei staining with propidium iodide in *N. schoberi* seeds.

Four variations of the experiment were performed in three replications:

1. *N. schoberi* seed + standard sample *Raphanus sativus* L. 'Saxa[32]' leaf + PVP.

2. *N. schoberi* seed + standard sample *Raphanus sativus* L. 'Saxa[32]' leaf with no added PVP.

3. Standard sample *Raphanus sativus* L. 'Saxa[32]' leaf + PVP.

4. Standard sample *Raphanus sativus* L. 'Saxa[32]' leaf with no added PVP.

After extraction of the nuclei the samples were filtered through a single-use Cellecrics Partec 50 micron Sysmex Partec filter, with the addition of 2 ml of staining solution containing the staining buffer (Sysmex Partec) propidium iodide (50 µg/ml) and RNAse A (50 µg/ml). Staining occurred at room temperature in a dark location for 40 minutes. The prepared samples were stored in a refrigerator for no more than four hours.

For each sample 15,000 events were collected (with a required overall number of 5,000 to 20,000) (Galbraith et al. 1997). The relative nuclear DNA content was calculated based on the linear relationship between fluorescent signals from the stained nuclei of the studied samples, and the internal standard.

Basic statistics (mean, median, standard deviation) were calculated in R (R Development Core Team 2011).

Analysis of the relative DNA content of *N. schoberi* seeds showed that with no PVP added to the extraction buffer, 2C values averaged 1.11±0.021 higher in comparison with experiment variations adding PVP. This is due to the fact that in the absence of an antioxidant the fluorescence of the nuclei isolated from standard leaf samples of *Raphanus sativus* L. 'Saxa[32]' decreases: G0/G1 and G2 peaks of the standard sample shift left by an average 2.5±0.043 channels on a 256-channel scale, while the fluorescence intensity of nuclei isolated from *N. schoberi* seeds does not change under the antioxidant effect (Figure 1a, b).

Similar patterns were revealed in the experiment where only standard *Raphanus sativus* L. 'Saxa[32]' leaves were analyzed – with no added PVP a decrease in fluorescence of the nuclei was observed and, accordingly, G0/G1 and G2 peaks shifted left by the value indicated above (Figure 1c, d).

Although the flow cytometry method used to analyze genome size in plants was simple and reliable, one of the most difficult problems encountered by the FCM users was adjusting the buffer composition for species containing staining inhibitors such as phenols, caffeine and other secondary metabolites in leaf cells (Jedrzejczyk & Sliwinska 2010). Notwithstanding the existence of research in this field (Price et al. 2000, Noirot et al. 2000, 2003), little information has been available to date on how stain inhibitors act, and there is no universal method to completely avoid their effect on assessment of DNA content in various organisms (Greilhuber et al. 2007, Greilhuber 2008). There is evidence that inhibition is associated with the intercalation of secondary metabolites in DNA and/or their direct reaction to a dye molecule, preventing its fluorescence (Loureiro et al. 2006, Loureiro et al. 2007). Adding antioxidants (polyvinylpyrrolidone, β-mercaptoethanol, dithiothreitol) to the buffer helps to rectify the situation; however, it does not always guarantee correct measurement of the DNA content, as the composition and concentration of secondary metabolites

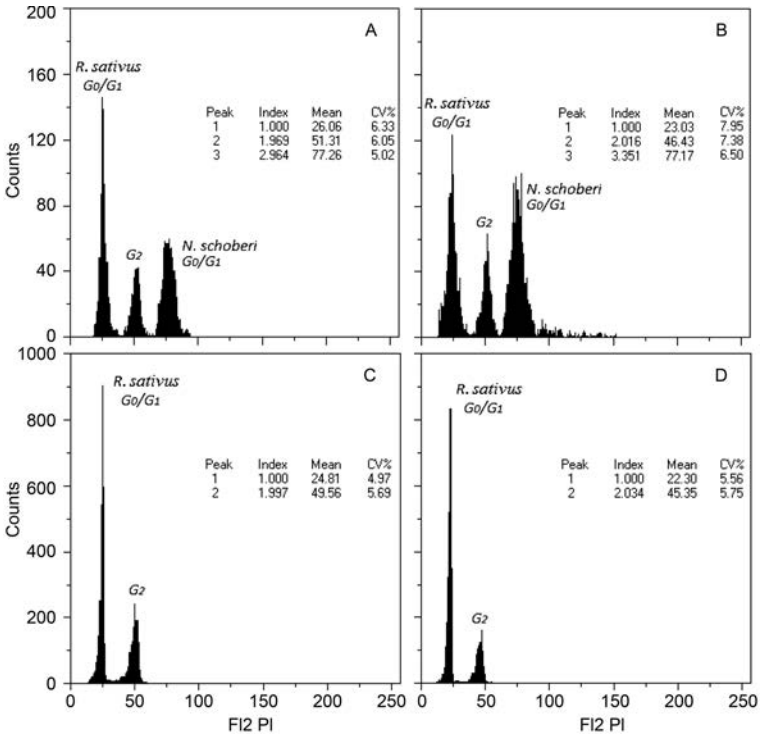

Figure 1 Histogram of PI fluorescence intensity: A – *N. schoberi* + standard *Raphanus sativus* L. 'Saxa[32]' + PVP; B – *N. schoberi* + standard *Raphanus sativus* L. 'Saxa[32]'; C – standard *Raphanus sativus* L. 'Saxa[32]' (with PVP addition); D –. standard *Raphanus sativus* L. 'Saxa[32]' (without PVP addition)

differ in various plant species and tissues (Yokoya et al. 2000, Sliwinska et al. 2005, Jedrzejczyk & Sliwinska 2010).

In this regard, for analysis of genome size it is proposed to use, where possible, plant organs free of inhibitors, particularly seeds (Sliwinska et al. 2005, 2006, 2009), which have a number of advantages over leaf material in terms of accessibility for research, ease of transportation, and capability of being stored over a long term. Mature embryos of dormant seeds usually contain most of the cells in the G0/G1 phase of the cell cycle (Sliwinska et al., 2009) and are thus suitable for determining the relative 2C DNA content. The presence of staining inhibitors should be checked for each species, especially woody ones, regardless of the plant material used for measurements, and the composition of buffer isolation nuclei should be optimized for plant species and their tissues (Jedrzejczyk & Sliwinska 2010).

The characteristics we found of the decrease in fluorescence of nuclei isolated from the leaves of a standard sample of *Raphanus sativus* L. 'Saxa[32]' with no polyvinylpyrrolidone added to the extracting buffer, indicate the presence of staining inhibitors in leaves. The absence of shifts of G0/G1 peaks of *N. schoberi* on the histogram of PI fluorescence intensity is evidence of the absence of inhibitor substances in *N. schoberi* seeds.

Thus, *N. schoberi* seeds do not contain substances inhibiting PI nuclei staining; they are sufficiently large (up to 1.5 cm in length and 0.5 cm in diameter), have no endosperm, and are easily transported and stored, which makes them a convenient material for use in cytometric studies.

ACKNOWLEDGEMENTS

The study was supported in part by the Russian Foundation for Basic Research (grant 16-04-00631 to E.V. Banaev). We express our thanks to Steve Kohl for his assistance in preparing the article.

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