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EVALUATION OF KARYOTYPE, GENOME SIZE AND GC CONTENT IN *DIANTHUS CHINENSIS* L.

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Abstract

Dianthus chinensis is an important ornamental species in the genus *Dianthus*. Its high tolerance to biotic and abiotic stresses is beneficial for genetic improvements of *Dianthus*. In this paper, we conducted a karyotype analysis on mitotic chromosomes of *D. chinensis*. Its genome size (2C value) and GC content were measured by flow cytometry using propidium iodide (PI) and 4', 6-Diamidino-2-phenylindole (DAPI) as dyes. The results showed that the *D. chinensis* has 2n = 2χ= 30 chromosomes. The chromosomes are metacentric or submetacentric. Their length varies from 1.60 ± 0.57 to 4.70 ± 0.85 µm. The estimation of its genome size is 772.53 ± 0.91 Mbp and GC content is 39.80 ± 0.12%. The cytological information of *D. chinensis* is helpful to clarify phylogenic relationships and assess potential plant transformation experiments among *Dianthus* species.

Key words: *Dianthus chinensis* L., Karyotype, Flow cytometry, Genomic size, GC content.

Introduction

Genome size or nuclear DNA content is essential for planning gene cloning and genome sequencing projects (Rabinowicz & Bennetzen, 2006). Flow cytometry (FCM) has become the most popular application for the estimation of genome size and ploidy level because of its convenience, speed and reliability (Balao *et al.*, 2009; Clarindo & Carvalho, 2011; Sakiroglu & Kaya, 2012). The amount of DNA in G1 phase 2n nuclei is referred to as the 2C-value (2C). It is measured as the mean mass or the mean number of base pairs of DNA in the nucleus. The estimation of DNA quantity (C-value) has led to the discovery of more than 2400-fold variation in the genome size in plants, from 61 Mbp in *Genlisea tuberosa* to 149 Gbp in *Paris japonica* (Bennett & Leitch, 2011; Andreas *et al*., 2014).

D. chinensis is indigenous to northern China, Korea, Mongolia, Kazakhstan and southeastern Russia, and is well adapted to many kinds of environments (Lim, 2014). Due to its early flowering and high tolerance to *Fusarium* wilt, drought- and salt- stress, *D. chinensis* is widely cultivated as an ornamental garden plant (Sparnaaij & Putten, 1990; Kantia & Kothari, 2002; He *et al*., 2012). It plays an important role on breeding new and desirable cultivars in the genus *Dianthus*. Although *D. chinensis* was mentioned to possess 2n = 2χ= 30 chromosomes (Fu *et al.*, 2008), the information of karyotype, genome size and GC content has not been studied much in detail. Therefore, the cytotype analysis of *D. chinensis* was carried out using chromosome counts and flow cytometry in our study.

Materials and Methods

Plant materials: *D. chinensis* seeds were gathered from the Daqingshan Mountain, located in Hohhot, Inner Mongolia, China, is about 2000 meters above the sea level. The seeds of *Brachypodium distachyon*, *Oryza sativa*, *Sorghum bicolor* and *Zea mays* were obtained from the Institute of Botany, Chinese Academy of Science, Beijing, China.

Chromosome counts: *D. chinensis* seeds were germinated in petri dishes at 15℃ in the dark. Root tips were excised when they were about 1 cm in length and pretreated with distilled water at 4℃ for 12 h. The pretreated root tips were immersed in 0.002 M 8-hydroxyquinoline for 3-5 h, and then fixed in an ethanol: acetic acid (3:1) solution at 4℃ for 24 h. The meristematic portion of the root tips were removed, and treated in 1M hydrochloric acid at 60℃ for 5 min and 45% ice acetic acid at room temperature (22±2℃) for 15 min. After being stained in a carbol fuchsin solution, the samples were placed on glass slides and squashed. The karyotype was determined by examining 20 well-spread metaphase plates. Photomicrographs of chromosomes at the mitotic metaphase were taken with the Olympus BX51 (Olympus Co., LTD, Japan). Chromosome measurements were performed using Image Pro Plus software. The chromosomes were classified based on the length and centromeric position, and arranged in order of decreasing length (*Levan et al.*, 1964).

Flow cytometry measures: Nuclear suspensions for flow cytometry were prepared from young true leaves of *B. distachyon*, *O. sativa*, *S. bicolor*, *Z. mays* and *D. chinensis* according to Doležel *et al*., (2007). For each species, 20 mg of true leaves were placed in the center of a clean plastic petri dish, and chopped in 1 mL nuclear isolation buffer (45 mM $MgCl_2$, 20 mM MOPS, 30 mM sodium citrate, 0.1% (v/v) Triton X-100, pH 7.0) on ice with a sharp razor blade. The resulting slurry was filtered through 42 µm nylon mesh into a labeled sample tube.

For DNA content, 50 µg mL^{-1} of Propidium Iodide (PI) simultaneously with 50 µg mL^{-1} of RNase were added to the filtrates. The suspensions of stained nuclei were passed through MoFlo XDP (Beckman Coulter, USA) with excitation / emission wavelengths of 488 nm/ 625 nm. The *D. chinensis* 2C value (DNA pg or Mbp) = Reference 2C value × (*D. chinensis* 2C mean peak position / reference 2C mean peak position) (Doležel *et al*., 2007).

For GC content, the filtrates were stained with 4 µg mL^{-1} of AT-selective 4', 6-Diamidino-2-phenylindole (DAPI). The suspensions of stained nuclei were passed through MoFlo XDP with excitation / emission wavelengths of 340

nm / 461 nm. The proportion of GC was determined according to Godelle *et al*., (1993): $\%GC_{D.chinensis} = 1 - \%AT_{reference} \times (R_{DAPI} / R_{PI})^{1/3}$, where R_{DAPI} = $intensity_{D.chinensis}$ / $intensity_{reference}$ for DAPI, R_{PI} = $intensity_{D.chinensis}$ / $intensity_{reference}$ for PI.

For each species, three independent measures were performed. All the reagents were bought from Sigma (USA).

Results

Chromosome counts: The adequate spread of mitotic figures is essential for a high degree of precision on measurements of chromosome size and a karyotype analysis. To get the adequate spread of mitotic figures in *D. chinensis*, two steps were modified in our study. Firstly, the germination temperature was lowered to 15℃. Seeds were normally germinated at room temperature (20-25℃) for chromosome preparation (Chen *et al*., 1998; Balao *et al*., 2009). Secondly, the fixed root tips were treated in 1 M hydrochloric acid at 60℃ for 5 min. The time shorter or longer than 5 min would affect the final results. The metaphase chromosomes and the karyotype analysis are shown in Fig. 1. The basic number in the genus *Dianthus* is $\chi = 15$ (Carolin, 1957). Therefore, the chromosome number of *D. chinensis* corresponds to diploid level ($2n = 2\chi = 30$). The length of metaphase chromosomes ranges from 1.60 ± 0.57 μm to 4.70 ± 0.85 μm. The karyotype consists of metacentric and submetacentric chromosomes.

Determination of suitable internal DNA reference standard: To avoid the influence on the staining intensity, such as dye concentration, ions present, pH or temperature, a suitable internal DNA reference standard is critical to FCM genome size studies (Doležel *et al*., 2007). The genome size of the referent plant does not differ from that of the unknown sample more than twofold (Doležel *et al*., 2007). The nuclear DNA contents of *B. distachyon*, *O. sativa*, *S. bicolor* and *Z. mays* are known and chosen as potential DNA reference standards (Table 1). Their relative fluorescence intensities of the stained nuclei were measured by FCM (Table 2). By checking peak symmetries and evaluating the distributions of fluorescence intensity (width of DNA peak), the relative fluorescence intensity ration between 2C *D. chinensis* (channel number 41.81) and 2C *O. sativa* (channel number 23.33) is 1.79. Such ratio is in accordance with suggestions by Doleže *et al*., (2007). The channel number for 2C *S. bicolor* is almost the same as the 2C *D. chinensis*. The ratio of the relative fluorescence intensity between *D chinensis* and *B. distachyon* is 3.53, and between *D chinensis* and *Z. mays* is 0.35. These ratios are not satisfied with the requirement of the internal standardization. Therefore, *O. sativa* is used as the internal standard in our study.

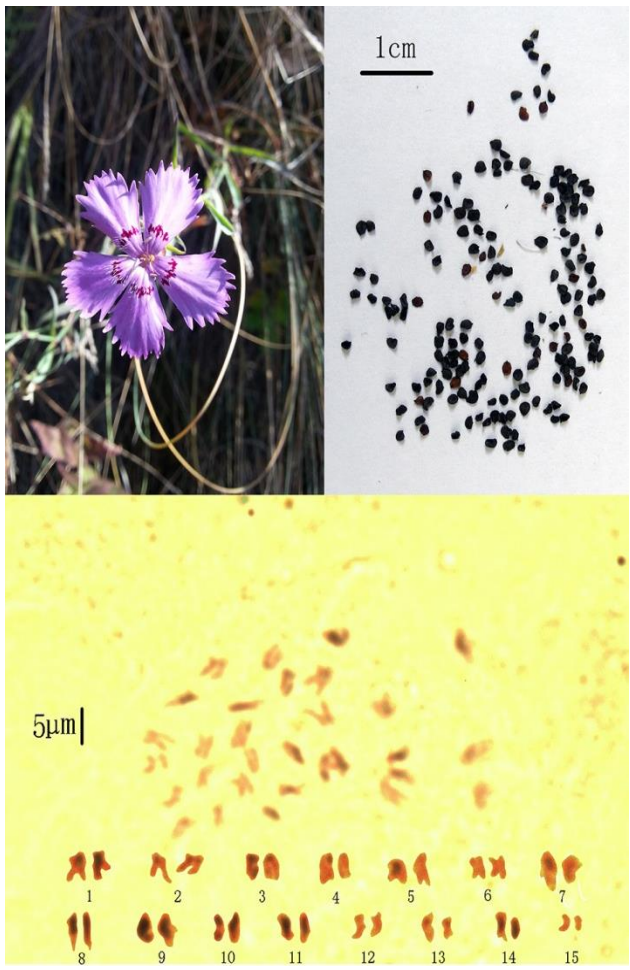

Fig 1. Flower, seeds and metaphase chromosomes of *D. chinensis*.

Evaluation of DNA content and GC percentage in D. chinensis: Nuclei of *D. chinensis* and *O. sativa* were stained with PI and run concurrently (Fig. 2). The histograms shows the best resolution level. Mean channel number of *O. sativa* is 28.45 ± 0.59 and of *D. chinensis* is 56.50 ± 1.12. The coefficient of variations (CV) for peaks ranges from 3.14% to 4.06%. The reference 2C value of *O. sativa* is 389 Mbp. Based on the formula (Doleže *et al*., 2007), the 2C value of *D. chinensis* is 772.53 ± 0.91 Mbp.

The GC content of *D. chinensis* was determined by PI and DAPI staining (Fig. 2). The GC content of *O. sativa* is 43.60% (International Rice Genome Sequencing Project, 2005). Its AT content calculated is 56.40%. Based on the formula (Godelle *et al*., 1993), the fluorescence intensity ratio between *D. chinensis* (channel number 238.17) and *O. sativa* (channel number 98.42) is 2.42 with DAPI staining and is 1.99 with PI staining. The CVs for peaks vary from 3.55% to 4.10%. Therefore, the GC content of *D. chinensis* is 39.80 ± 0.12%.

Table 1. Nuclear DNA content of potential reference standards.

Species	Chromosome numbers	2C nuclear DNA (Mbp)
B. distachyon	$2n = 2\chi = 10$	272 (The international brachypodium initiative, 2010)
O. sativa	$2n = 2\chi = 24$	389 (International rice genome sequencing project, 2005)
S. bicolor	$2n = 2\chi = 20$	734 (Paterson *et al*., 2009)
Z. mays	$2n = 2\chi = 20$	2300 (Schnable *et al*., 2009)

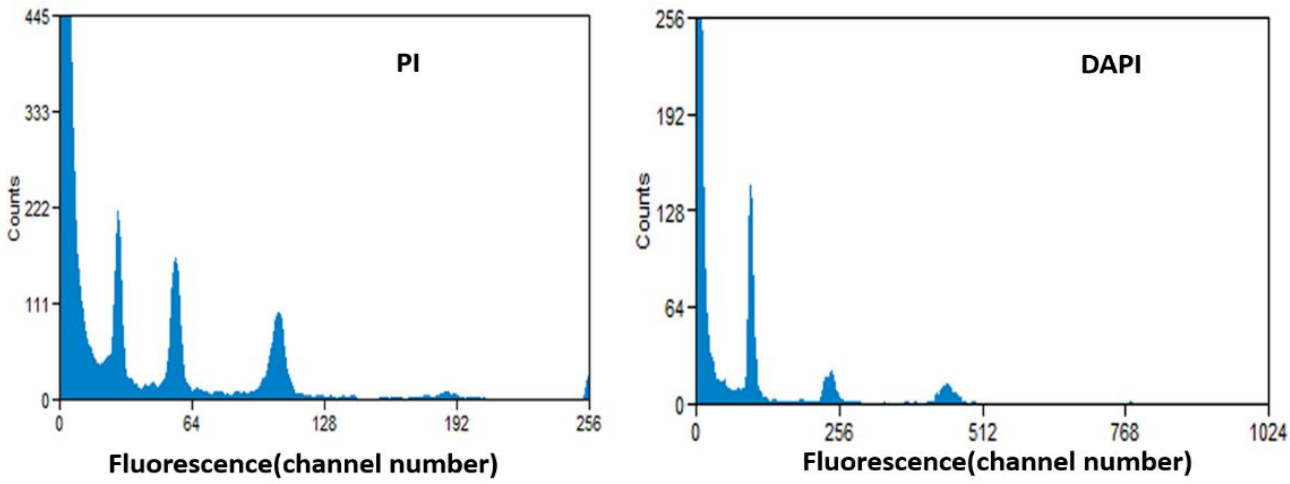

Fig 2. Histograms of fluorescence intensities measured by FCM. Left Histograms showing G_0/G_1 peaks with cv ranging from 3.14% to 4.06% obtained from PI-stained nuclear suspension prepared from leaves of *O. sativa* (internal standard, channel 28.45, 775 nuclei) and *D. chinensis* (channel 56.50, 848 nuclei). Right Histograms showing G_0/G_1 peaks with cv ranging from 3.55% to 4.10% obtained from DAPI-stained nuclear suspension prepared from leaves of *O. sativa* (internal standard, channel 98.42, 1540 nuclei) and *D. chinensis* (channel 238.17, 552 nuclei).

Table 2. Fluorescence intensity of propidium iodide-stained nuclei in diploid of different species.

Species	Mean channel number	CV%
B. distachyon	11.85	4.16
O. sativa	23.33	4.71
S. bicolor	40.28	3.50
Z. mays	119.34	3.44
D. chinensis	41.81	4.20

Discussion

Carolin (1957) studied ploidy levels in 91 species of the genus *Dianthus* and found 67% of them were diploid. A more comprehensive understanding of the karyotypes will help clarify the phylogenic relationships and assist in strategic assessment of potential plant transformation experiments among the species in *Dianthus* (Chen *et al.*, 1998). By improving chromosome preparation techniques, we get the adequate spread of mitotic figures and describe the karyotype of *D. chinensis*. *D. chinensis* is diploid and possesses 2n = 2χ= 30 chromosomes, which is in accordance with Fu *et al*., (2008). Metaphase chromosomes of *D. chinensis* are longer than those of *D. broteri* (Balao *et al.*, 2009).

Information of nuclear DNA amounts has been used in the prediction of cell size and stomatal density (Beaulieu *et al.*, 2008), the pattern of genome size evolution (Balao *et al.*, 2009; Clarindo & Carvalho, 2011; Renny-Byfield *et al.*, 2013; Sharma *et al.*, 2019), and the separation of closely related species and their hybrid in plants (Mahelka *et al.*, 2005). To measure nuclear DNA amounts or estimate DNA C-values in plants, the popular method available to botanists is FCM (Sliwinska *et al.*, 2018; Bainard *et al.*, 2019), and it will be the dominant method of choice in the future due to its high precision (Bennett & Leitch, 2011; Bourge *et al.*, 2018). By FCM, the nuclear DNA content of carnation (2n = 2χ= 30) is 670 Mb (Yagi *et al.*, 2014). By genome sequencing, the genome size of carnation cultivar 'Francesco' is 622 Mb (Yagi *et al*., 2014), which falls into the range measured by FCM. The estimation of the DNA content in *D. chinensis* (2n = 2χ = 30) is 772.53 Mb by FCM, which is larger than that in carnation. The genome size of *D. chinensis* estimated by FCM provides a reference for genome sequencing in the future. The variation in 2C is associated with variations in a number of other functional traits and environmental variables (Knight & Ackerly, 2002). *D. chinensis* and carnation belong to the genus *Dianthus*. Due to its high tolerance to biotic and abiotic stresses, *D. chinensis* can be intercrossed with carnation for new and desirable cultivars.

GC levels are related to natural selection. In different GC levels, new insights of major composition transition of genome, evolution of coding sequence and bias in codon usage are obtained in the field of evolutionary genomics (Leushkin *et al.*, 2013; Veleba *et al.*, 2014). Compared with the GC content of carnation (36%) (Yagi *et al.*, 2014), the high GC content (39.80 ± 0.12%) is found in *D. chinensis*. The estimation of the GC content is within the range (from 33% to 50%) of the entire known genomic GC content variation in vascular plants (Šmarda & Bureš, 2012). Genomic % GC is found to have a positive correlation with genome size, which means that large genomes are often GC-rich (Veleba *et al.*, 2014). Compared with genome information of carnation, large genome size of *D. chinensis* has rich GC content. In order to facilitate *D. chinensis* in the horticultural industry and breeding program, further researches should be conducted on the genome differences of structural and functional properties between *D. chinensis* and carnation.

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