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Original Article

Nuclear DNA content of the pigeon orchid (*Dendrobium crumenatum* Sw.) with the analysis of flow cytometry

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Abstract

Nuclear DNA content for the adult plants grown in a greenhouse and *in vitro* young plantlets of the pigeon orchid (*Dendrobium crumenatum* Sw.) was analyzed using flow cytometry. The resulting 2C DNA values ranged from 2.30±0.14 pg to 2.43±0.06 pg. However, nuclear DNA ploidy levels of long-term *in vitro* plantlets were found to be triploid and tetraploid. These ploidy levels were confirmed by chromosome counting. Tetraploid individuals (2n = 4x = 76) had approximately two times DNA content than diploid (2n = 2x = 38) individuals. This variation may be due to prolonged cultivation and the presence of exogenous plant growth regulators.

Keywords: *Dendrobium crumenatum* Sw., DNA content, flow cytometry, *in vitro* culture, pigeon orchid

1. Introduction

The genus *Dendrobium* is a member of Orchidaceae which is considered the largest plant family. *Dendrobium* is of considerable interest due to its broad geographic distribution and high value as a commercial commodity. The pigeon orchid, *Dendrobium crumenatum* Sw., is a tropical epiphytic orchid and has many uncommon features such as fragrant white flower and gregarious flowering (for review see Meesawat and Kanchanapoom, 2007). Classification of the orchid species using morphology as well as chromosome counting is dynamic (Jones *et al.*, 1998). Considering the difficulties with the classical taxonomy, the use of flow cytometry to analyze either ploidy level or nuclear DNA content has been employed in many plant species such as *Sesleria* (Lysak and Dolezel, 1998), Macaronesian angiosperms (Suda *et al.*, 2003) and oil palm (Srisawat *et al.*, 2005). Flow cytometry is a technique which allows a simple, rapid, accurate and convenient method for determination of nuclear DNA content (Dolezel, 1991). In addition, the method requires only a small amount of tissue and is therefore non-destructive. To date the DNA content of orchids is reported for only 41 species (Jones *et al.*, 1998). Therefore, the present study was undertaken to investigate the DNA content of the selected pigeon orchid from both natural and *in vitro* cultured plants.

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2. Materials and Methods

2.1 Plant material

Leaf samples of naturally grown *D. crumenatum* were collected from three sources namely Songkhla, Pattani, and Narathiwat Provinces of southern Thailand. They were kept under greenhouse conditions at the Biology Department, Prince of Songkla University. In addition, leaves of plantlets derived from two *in vitro* culture systems were analyzed. In the first *in vitro* culture system, plantlets were obtained from the method as described by Meesawat and Kanchanapoom (2002). Briefly, *Dendrobium* buds were cultured on modified Vacin and Went medium to initiate callus. This callus was converted into protocorm like bodies (PLBs) and plantlets were subsequently regenerated from these PLBs. In the

second *in vitro* culture system, PLBs thus obtained was transferred to a liquid Knudson medium and plantlets were regenerated following the method as described by Meesawat and Kanchanapoom (2007).

2.2 Nuclei extraction

Nuclei for DNA content analysis were extracted from fully expanded second young leaves. Extraction of nuclei and DNA staining were performed according to Pfosser *et al.* (1995). Approximately 20-30 mg of tissues were finely chopped using a sharp razor blade into 0.2-0.5 mm in a 1 ml Tris-$MgCl_2$ extraction buffer containing 0.2M Tris, 4 mM $MgCl_2$, 0.5%(w/v)Triton X-100 and 3% (w/v) polyvinyl-pyrrolidone. After extraction, 50μl of RNase and propidium iodide were added immediately prior to filter through a 42 μm nylon mesh. After filtration, samples were stored on ice prior to analysis.

2.3 Flow cytometric analysis

The fluorescence of a minimum of 5000 propidium iodide-stained nuclei was estimated using a flow cytometer FACScalibur Becton Dickinson (Scientific Equipment Center, Prince of Songkla University). The internal reference standard *Raphanus sativus* L. cv. Saxa nova (2C = 1.11 pg) was kindly provided by Dr Jaroslav Dolezel, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Czech Republic. During analysis the reference standard peak was positioned at channel 200 of relative fluorescence intensity for instrument calibration. Propidium iodide was measured at 585 nm to read 2C nuclei DNA content of sample. The obtained histograms were computerized by CellQuest software packages (Becton Dickinson, USA). The sample 2C DNA content was calculated according to the formula

Dendrobium 2C DNA content

$$= \frac{\textit{Dendrobium} \text{ peak mean}}{\textit{Raphanus} \text{ peak mean}} \times \text{2C DNA of } \textit{Raphanus}$$

The number of base pairs per haploid genome was calculated based on the equivalent of 1 pg DNA = 965 mega-base pair (Bennett and Smith, 1976).

2.4 Chromosome counts

Observations were made on root tips (0.5 cm in length) of greenhouse and *in vitro* grown plants. Samples were pretreated in 0.02% colchicine for 4 hours and fixed in Carnoy's fluid (3 parts 95% ethanol and 1 part glacial acetic acid) for 24 hours and stored in 70% ethanol in refrigerator (12°C). This treatment was followed by hydrolysis in 1N HCl at 60°C for 10-15 min. The root tip cuttings were squashed by Feulgen method (Sharma and Sharma, 1980) and examined 1000x magnification under the light microscope (Olympus model CH 30). Photographs were taken at 100x magnification using an attached Olympus DP11 camera.

2.5 Statistical analysis

All experiments were carried out at least 3 times with 5-10 replicates per treatment. The fluorescence histograms were resolved into G0/G1 (2C), S and G2/M (4C) cell-cycle compartments with a peak-reflect algorithm using two Gaussian curves (WinMDI version 2.8). DNA content of the pigeon orchid was statistically analyzed by ANOVA (analysis of variance) at $p \leq 0.05$. Tukey test was performed for routine multiple mean comparison.

3. Results

Table 1 shows the mean 2C DNA content of *D. crumenatum* from natural grown plants ranged from 2.36± 0.14 pg $2C^{-1}$ (Pattani source) to 2.43 ± 0.06 pg $2C^{-1}$ (Songkla source). No differences in 2C DNA content were found ($p \leq 0.05$) between plants grown in a greenhouse and the first *in vitro* culture system derived plants (2.30±0.14 pg $2C^{-1}$). In contrast, the second *in vitro* culture system derived plants showed 2C DNA content with a different 2C DNA content level (4.90±0.03 pg $2C^{-1}$) ($p \leq 0.05$). 1C nuclear DNA content expressed in mega-base pairs obtained in this study had the same trend as 2C DNA content nuclei. All analyzed plants grown in a greenhouse and the first system *in vitro* derived plants of *D. crumenatum* were found to be diploid with 2n = 2x = 38 (Table 1, Figure 1A). This finding confirmed that all plants using flow cytometry were indeed diploid thus chromosomal level in both types were stable.

On the other hand, flow cytometry analyses could easily detect triploid and tetraploid cells in regenerated plants derived from the second *in vitro* culture system, and mean 2C DNA contents were 3.40±0.00 pg $2C^{-1}$ and 4.90±0.03 pg $2C^{-1}$ nuclei for triploid and tetraploid plants, respectively (Table 1, Figure 2). These triploid and tetraploid plants had normal morphology; however, triploid plants exhibited long and light green leaves while tetraploid plants had thicker and darker green leaves (Figure 3A, B). To confirm the tetraploid cells of the second *in vitro* culture system derived plants, mitotic chromosome behavior showed chromosome number of 2n = 4x = 76 in tetraploid individuals (Figure 1B).

4. Discussion

Dendrobium is the largest orchid genus comprising approximately 1000 identified species (Dressler, 1981). To date, the DNA content is known for 37 *Dendrobium* species (Jones *et al.*, 1998). Determination of DNA content previously reported in other plant species including orchids was carried out by several methods i.e. reassociation kinetics (Nagl and Capesius, 1977), Feulgen microdensitometry (Narayan *et al.*, 1989), fluorescent microdensitometry

Table 1. Chromosome number, mean DNA content with standard error in picograms and mega base pairs for *D. crumenatum* from different sources.

D. crumenatum	Chromosome number	DNA content (pg) (mean ± SE)	Mbp
Natural sources			
1. Songkhla province	38	2.43 ± 0.06[a]	1172[a]
2. Pattani province	38	2.36 ± 0.14[a]	1138[a]
3. Narathiwat province	38	2.42 ± 0.12[a]	1167[a]
In vitro sources			
1. The 1st *in vitro* culture system*	38	2.30 ± 0.14[a]	1109[a]
2. The 2nd *in vitro* culture system**	57	3.40 ± 0.00[b]	1641[b]
3. The 2nd *in vitro* culture system**	76	4.90 ± 0.03[c]	2364[c]

The different letters within column show significant difference of nuclear DNA content (mean ± SE) analyzed by Tukey test at $p \leq 0.05$.
Mbp = mega-base pair (1 pg DNA = 965 mega-base pairs)
* Meesawat and Kanchanapoom (2002)
** Meesawat and Kanchanapoom (2007)

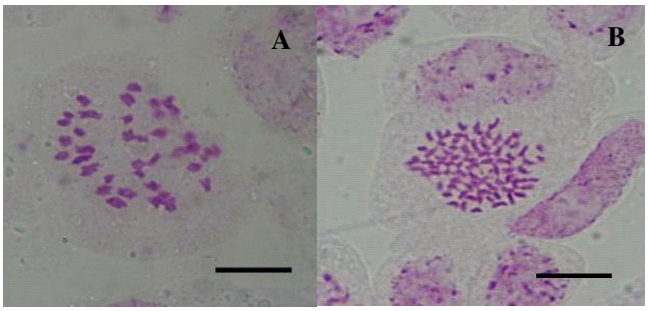

Figure 1. Mitotic metaphase of root tips showing diploid 2n = 2x = 38 chromosomes (A) and tetraploid 2n = 4x = 76 chromosomes (B). Bar = 10 mm.

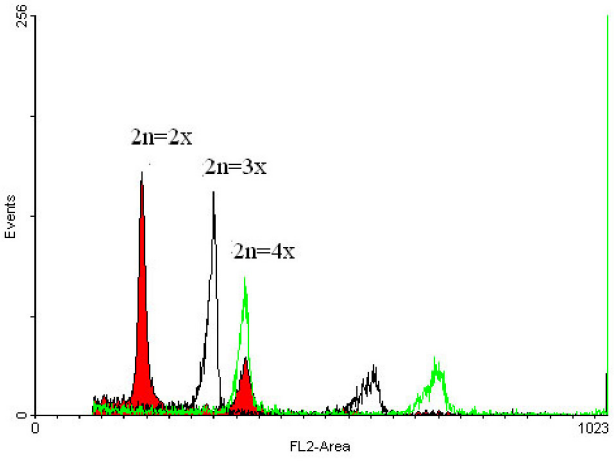

Figure 2. Histogram of fluorescence intensity of *D. crumenatum* nuclei stained with propidium iodide. Results represent measurements of diploid (2n = 2x), triploid, (2n = 3x), and tetraploid (2n = 4x) plants.

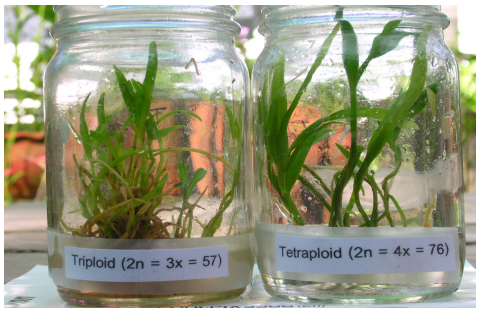

Figure 3. Triploid (A) and tetraploid (B) plantlets of *D. crumenatum* cultured *in vitro*.

(Bennett and Leitch, 1995) and flow cytometry (Jones *et al.*, 1998). The present study presents 2C DNA content of *D. crumenatum* as determined by flow cytometry. Flow cytometry can be a very useful method for the rapid detection of DNA content and ploidy level variation. DNA content from this study yielded the value of 2.43±0.06 pg $2C^{-1}$ which is different from the finding of Jones *et al.* (1998) of 2.61±0.17 pg $2C^{-1}$despite uniform number of 2n = 2x = 38. The difference in DNA content could occur due to the type of laser lamp equipped in the flow cytometer (Dolezel *et al.*, 1998).

Intraspecific variation did not exist among the wild *D. crumenatum* collected from three different sources and the first *in vitro* culture system since adult plants grown in a greenhouse and *in vitro* young plantlets were both found to be diploid (2n = 2x = 38). However, differences in DNA content were found for the second *in vitro* culture system and regenerants were found to be triploid (2n = 3x = 57) and tetraploid (2n = 4x = 76). Long-term cultivation and the presence of exogenous plant growth regulators are suspected to induce genetic instability *in vitro* (Thiem and Sliwinska, 2003). Results from this study confirmed the usefulness of

flow cytometry as an efficient method for screening ploidy status in *Dendrobium* micropropagated plants and the results obtained by flow cytometry are confirmed by chromosome counts.

To conclude, this study provided the nuclear DNA content values for *D. crumenatum.* These values clearly demonstrated stability and were found to be an effective estimation of ploidy level in greenhouse-grown plants and tissue-culture derived plants despite its long term subculture and the presence of growth regulators.

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