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# Specific Differences in Nuclear DNA Content in the Genus *Cyclamen*

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## ABSTRACT

Relative nuclear DNA contents in *Cyclamen* species were estimated by flow cytometry with 4', 6-diamidino-2-phenylindole (DAPI) staining and propidium iodide (PI) staining. The relative fluorescence intensity (RFI) values in each species ranged from 1.521 to 8.071 and from 1.485 to 7.941 in flow cytometry with DAPI and PI staining, respectively. The ratio of RFI with PI staining to that with DAPI staining ranged from 0.93 to 1.15. Species belonged to the same subgenus indicated almost the same ratio of RFI with PI staining to that with DAPI staining. Subgeneric difference of the ratio was also observed. Flow cytometry seemed to be useful for the identification for interspecific hybrids in *Cyclamen* species, because the RFIs of interspecific hybrids were intermediate between that of their maternal and paternal species. The results of the present study should indicate that flow cytometry might be one of the effective tools for classification and identification of interspecific hybrids in the genus *Cyclamen*.

**Keywords:** cyclamen, 4',6-diamidino-2-phenylindole (DAPI), flow cytometry, interspecific hybrid, propidium iodide (PI)
**Abbreviations: DAPI**, 4',6-diamidino-2-phenylindole; **PI**, propidium iodide; **RFI**, relative fluorescent intensity

## INTRODUCTION

Cyclamen (*Cyclamen persicum* Mill) is cultivated throughout the temperate zone, and one of the most famous and important commercial ornamental plants in many countries. Only *C. persicum* has been used as the major commercial plant in the genus to the present, whereas the genus *Cyclamen* consists of 22 species (Grey-Wilson 2002).

It is difficult to produce interspecific hybrid seeds in most of the interspecific crosses in the genus because of the cross incompatibility, especially in the crosses between *C. persicum* and other species. Thus, *C. persicum* has not hybridized to other *Cyclamen* species in the wild (Jalali *et al.* 2012). Interspecific hybrids from the crosses between *C. persicum* and some wild species were, however, produced by ovule (or ovary) culture (Ishizaka and Uematsu 1990, 1992, 1995a; Ishizaka 1996; Ewald 1996; Sibusawa and Ogawa 1997; Takamura *et al.* 2002). By using interspecific hybridization in *Cyclamen* it can be expected to breed new cultivars with novel and useful characteristics such as the fragrance. Since hybrid plants take a long term to mature, early identification of interspecific hybrids is desirable.

Flow cytometry is one of the rapid methods for measuring nuclear DNA content, and can be performed by using tissue of young plants. Takamura and Yoshimura (2007) indicated that rapid determination of ploidy levels in cyclamen was possible by flow cytometry with DAPI staining. Possibility of the flow cytometric analysis as an early method for identifying interspecific hybrids between *C. persicum* and some other species was suggested (Ewald 1996; Takamura *et al*. 2001). PI as well as DAPI is used as staining chemicals in flow cytometric analysis. Buitendijk *et al*. (1997) and Mishiba *et al*. (2000) reported specific difference of the ratio of RFI with PI staining to that with DAPI staining in the genus *Alstroemeria* and *Calibrachoa*, respectively. No report on the specific difference in the genus *Cyclamen* is, however, available.

Relative nuclear DNA contents in 11 wild *Cyclamen* species to those in commercial cyclamen were, therefore, estimated by using flow cytometry both with DAPI staining and PI staining in the present study. The possibility and value of flow cytometric analysis as an index for classification and as a tool for identification of the interspecific hybrids in the genus *Cyclamen* was discussed.

## MATERIALS AND METHODS

### Flow cytometry by using DAPI and PI in 11 Cyclamen species

Eleven *Cyclamen* species listed in **Table 1** and *C. persicum* 'Largo' were used, whereas 'Largo' was used as an internal standard for flow cytometric analysis. The prominent peak of 'Largo' in flow cytometry has indicated 2C level of *C. persicum* (Takamura and Yoshimura 2007). All the plants were grown in the greenhouse of Kagawa University at natural temperature.

Nuclear samples were prepared from the sections of fresh young leaves (about 49 $mm^2$) of 11 *Cyclamen* species with the leaf section of 'Largo' as an internal standard. The leaf section of a sample and an internal standard were chopped altogether with a razor blade in a plastic Petri dish after adding a few drops of commercial buffer solution (solution A of plant high resolution DNA kit type P, Partec, Germany) for extracting nuclear DNA, according to the previous reports (Galbraith *et al*. 1983; Mishiba *et al*. 2000). The extract was incubated for 10 min at room temperature, and then filtered by using 20 μm nylon mesh.

As the staining solution, both DAPI and PI solution were used. DAPI solution was prepared according to Mishiba *et al*. (2000), whereas commercial buffer solution (CyStain PI absolute P staining buffer, Partec, Germany) was used for PI staining. The filtrate was increased the quantity by five times with the staining solution, and then incubated for 10 min and 30 min at room temperature in DAPI and PI staining, respectively. Fluorescent intensity of the nuclei in the filtrate was measured flow cytometric (PA-II, Partec, Germany). Five samples in each species were used in each experiment. At least 1000 cells were counted for flow cytometry in all samples, whereas more than 2000 cells were counted in almost all samples. The mean RFI values and the standard errors were calculated.

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**Table 1** Relative fluorescence intensities of eleven *Cyclamen* species obtained by PI and DAPI staining.

| Subgenus | Species | No. of plants investigated | Relative fluorescence intensity [z] | | PI/DAPI ratio [y] |
|---|---|---|---|---|---|
| | | | PI | DAPI | |
| *Psilanthum* | *C. creticum* | 5 | 2.204 ± 0.016 | 2.053 ± 0.025 | 1.07 |
| | *C. repandum* | 5 | 2.079 ± 0.031 | 1.933 ± 0.016 | 1.08 |
| *Gyrophoebe* | *C. alpinum* | 5 | 7.941 ± 0.117 | 8.071 ± 0.075 | 0.98 |
| | *C. coum* | 5 | 4.660 ± 0.051 | 4.496 ± 0.013 | 1.04 |
| | *C. pseudibericum* | 5 | 6.868 ± 0.057 | 6.627 ± 0.038 | 1.04 |
| | *C. cilicium* | 5 | 6.991 ± 0.070 | 6.617 ± 0.107 | 1.06 |
| | *C. mirabile* | 5 | 4.558 ± 0.032 | 4.316 ± 0.028 | 1.06 |
| *Cyclamen* | *C. hederifolium* | 5 | 1.689 ± 0.004 | 1.521 ± 0.011 | 1.11 |
| | *C. purpurascence* | 5 | 2.077 ± 0.019 | 1.806 ± 0.009 | 1.15 |
| *Eucosme* | *C. graecum* | 5 | 1.485 ± 0.012 | 1.536 ± 0.011 | 0.97 |
| | *C. rohlfsianum* (4*x*) | 4 | 1.679 ± 0.008 | 1.751 ± 0.011 | 0.96 |
| | *C. rohlfsianum* (6*x*) | 1 | 2.370 | 2.552 | 0.93 |

[z] Fluorescence intensity of a sample / Fluorescence intensity of an internal standard (*C. persicum* 'Largo')
[y] The ratio of relative fluorescence intensity with PI staining to that with DAPI staining

## Flow cytometry of interspecific hybrid by using PI staining

$F_1$ progenies obtained by *C. persicum* 550 (strain number) × *C. hederifolium* and *C. persicum* 'Golden Boy' × *C. purpurascens* crosses, grown in the greenhouse of Kagawa University, were used. Their parent plants were also used as the plant materials. Nuclear samples were prepared from the sections of fresh young leaves (about 25 $mm^2$). The leaf section of an interspecific hybrid and the sections of its parent plants were chopped altogether with a razor blade in a plastic Petri dish. Only PI staining solution was used in this experiment. RFI of isolated nuclei were investigated by flow cytometry. Two hybrids in each cross combination were used. More than 1500 cells were counted in all samples.

## RESULTS AND DISCUSSION

In almost all plants, a nuclei sample isolated from their leaves showed a small peak, probably corresponding to nuclei $G_2$/M stage, as well as a prominent peak of nuclei in $G_0/G_1$ stage (**Fig. 1**). These two peaks are observed in both DAPI and PI staining, and fluorescent intensity of the prominent peak was used for the calculation of RFI of plants in the 11 *Cyclamen* species to *C. persicum* 'Largo'.

All the plants in the same species showed roughly the same RFI value in both DAPI and PI staining except *C. alpinum* and *C. rohlfsianum* (**Fig. 2**). In *C. alpinum*, a plant showed little smaller RFI value with PI staining than other plants. A plant of *C. rohlfsianum* showed one and a half times larger RFI value than other *C. rohlfsianum* plants. Ishizaka *et al*. (2009) observed both tetraploid and hexaploid forms in *C. rohlfsianum*. The plant should be, therefore, hexaploid, while the other *C. rohlfsianum* plants were tetraploid. Specific difference of the RFI values in 11 *Cyclamen* species was detected in flow cytometry with PI staining as well as that with DAPI staining (**Table 1**). The values in each species ranged from 1.521 to 8.071 and from 1.485 to 7.941 in flow cytometry with DAPI and PI staining, respectively. This wide variation of RFI might be due to the variation of chromosome number (Grey-Wilson 2002) and/or chromosome size (Legro 1959).

The RFI values show that distinction by flow cytometry in 11 *Cyclamen* species will be possible in many cases, except between *C. pseudoibericum* and *C. cilicium*. Although the distinction between *C. hederifolium* and *C. rohlfsianum* (4*x*) by using flow cytometry with PI staining was difficult, the distinction with DAPI staining should be possible. On the other hand, the RFI value in *C. hederifolium* could be distinguished from that in *C. graecum* by flow cytometry with PI staining, whereas the difference of RFI value between *C. hederifolium* and *C. graecum* by flow cytometry with DAPI staining was not observed. Although both PI and DAPI are familiar chemicals for staining nucleus DNA in flow cytometry, the properties in staining DNA are different. DAPI is known to stain preferentially adenine-thymine rich regions of DNA (Manzini *et al*. 1983), while PI stains DNA

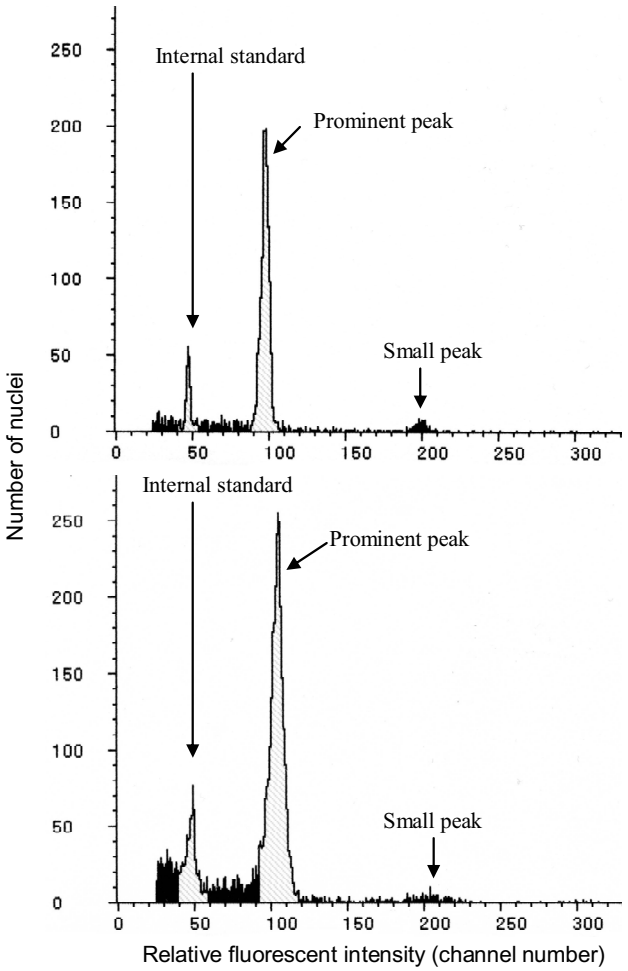


**Fig. 1 Typical flow cytometric histograms by using DAPI (upper) and PI (lower) staining for nuclei isolated from leaves of *C. creticum*.**

irrespective of the base composition (Le Pecq and Paoletti 1967). The different tendency of the RFI values between DAPI and PI staining should result from the different properties in staining DNA between DAPI and PI.

The different properties between DAPI and PI can be used for estimation of the difference in base composition in DNA. The low ratio of RFI with PI staining to that with DAPI staining should mean that the plant has DNA with more adenine-thymine rich regions. Buitendijk *et al*. (1997) showed the difference of PI/DAPI ratio between the Brazilian species and that of the Chilean species in the genus *Alstroemeria*. Mishiba *et al*. (2000) also suggested the possibility of discrimination between *Petunia sensu* and some *Calibrachoa* species by using the PI/DAPI ratio. Specific difference in the PI/DAPI ratio in 11 *Cyclamen* species was

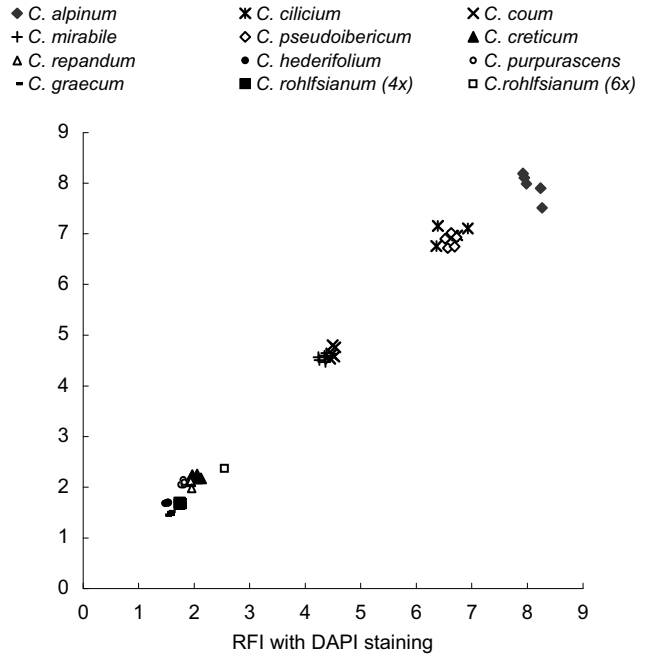


**Fig. 2 Scatter diagram of relative fluorescence intensities (RFI) stained with PI and DAPI in the plants of 11 *Cyclamen* species.**

observed, and the ratio ranged from 0.93 to 1.15 as shown in **Table 1**. Species belonged to the same subgenus, reported by Schwarz (1964), showed almost the same the PI/DAPI ratio. Although the PI/DAPI ratio in *C. alpinum* was lower than other species in the subgenus *Gryophoebe*, this should be resulted from little smaller RFI with PI staining of a plant as mentioned above. Subgeneric difference of the ratio was also observed, as species in the subgenus *Eucosme* has DNA with more adenine-thymine rich regions and with less guanine-cytosine rich regions as compared to species in other subgenus. These results suggest that the PI/DAPI ratio have a possibility to be one of the index character discriminating taxa in the genus *Cyclamen*.

Ishizaka and Uematsu (1992, 1995) and Ishizaka (1996) obtained progenies between cyclamen cultivars (*C. persicum*) and the wild relatives (*C. hederifolium*, *C. purpurascens*, *C. glaecum*) by using ovule culture, and identified them as interspecific hybrids by observing their fertility, and chromosomal and morphological characteristics. However, observation of the chromosomal characteristics of plants in the genus *Cyclamen* is not easy because of the small cell- and chromosome size. An early and easy method for identification of interspecific hybrids in the genus *Cyclamen* is, therefore desirable.

The RFI values with PI staining in $F_1$ (550 × *C. hederifolium*) and $F_1$ ('Golden Boy'× *C. purpurascens*) were intermediate between their seed and pollen parent (**Fig. 3**). Ewald (1996) indicated that flow cytometry with DAPI staining was suitable for an early identification of interspecific hybrid between *C. persicum* and *C. purpurascens*. Takamura *et al.* (2001) also indicated the possibility of early and rapid identification by using flow cytometry with DAPI staining for interspecific hybrids between *C. persicum* and *C. hederifolium* as well as those between *C. persicum* and *C. purpurascens*. Results of the present study suggest that flow cytometry with PI staining as well as that with DAPI staining can be used as a method for early and rapid identification of interspecific hybrid in the genus *Cyclamen*. This information should be useful, because specific difference of tendency of the RFI values between DAPI and PI staining in the genus *Cyclamen* was observed as mentioned above. Early identification of hybrids by flow cytometry with PI staining in interspecific crosses between *C. hederifolium* and *C. graecum* might be possible, whereas the identification by flow cytometry with DAPI staining in the crosses might be difficult.

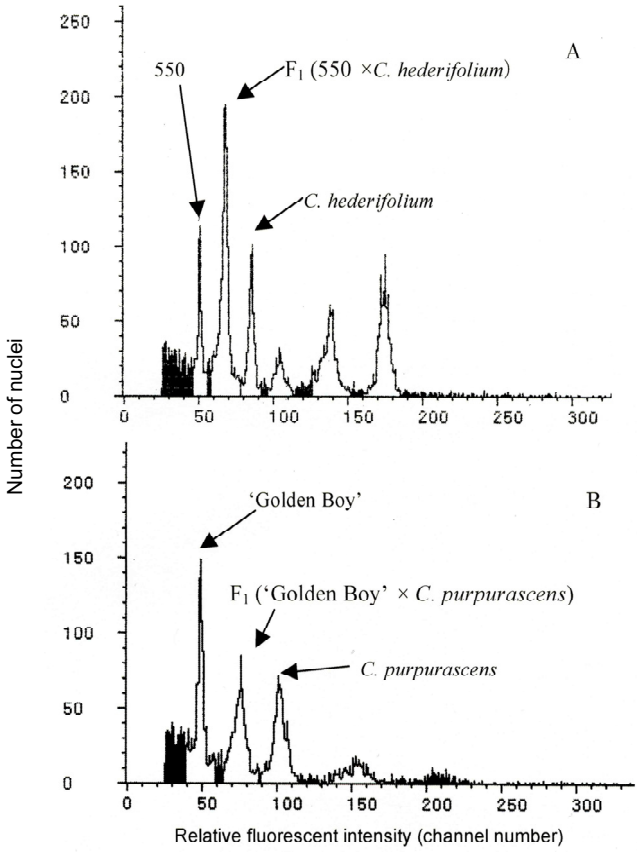


**Fig. 3 Typical flow cytometric histograms by using PI staining for nuclei isolated from leaves of inter-specific hybrids and their parents.** A: $F_1$ (*C. persicum* 550 × *C. hederifolium*); B: $F_1$ (*C. persicum* 'Golden Boy' × *C. purpurascens*).

## CONCLUSIONS

Thus, specific difference of DNA content and subgeneric difference of the PI/DAPI ratio, which should be related the ratio of adenine-thymine rich regions of DNA to the guanine-cytosine rich regions, in the genus *Cyclamen* was observed by flow cytometry with PI and DAPI staining in the present study. These results indicate that flow cytometry can be an effective tool for classification and identification of interspecific hybrids in the genus *Cyclamen*.

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