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Nuclear DNA Contents of *Phalaenopsis* sp. and *Doritis pulcherrima*

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Abstract. **Nuclear DNA contents were estimated by flow cytometry in 18 *Phalaenopsis* Blume species and *Doritis pulcherrima* Lindl. DNA amounts differed 6.07-fold, from 2.74 pg/diploid nuclear DNA content (2C) in *P. sanderiana* Rchb.f. to 16.61 pg/2C in *P. parishii* Rchb.f. Nuclear DNA contents of *P. aphrodite* Rchb.f. clones, W01-38 (2n = 2x = 38), W01-41 (2n = 3x = 57), and W01-22 (2n = 4x = 76), displayed a linear relationship with their chromosome numbers, indicating the accuracy of flow cytometry. Our results also suggest that the 2C-values of the *Phalaenopsis* sp. correlate with their chromosome sizes. The comparative analyses of DNA contents may provide information to molecular geneticists and systematists for genome analysis in *Phalaenopsis*. Endoreduplication was found in various tissues of *P. equestris* at different levels. The highest degree of endoreduplication in *P. equestris* was detected in leaves.**

Phalaenopsis Blume sp. are among the most beautiful of all orchids. The genera *Phalaenopsis* and *Doritis* Sw. belong to the Orchidaceae, subfamily Epidendroideae, tribe Vandeae, subtribe Aeridinae. *Phalaenopsis* comprises over 45 species in nine sections indigenous throughout most of Asia (Sweet, 1980). These species are distributed from India, south China, Thailand, Malaysia and Indonesia to the Philippines, New Guinea, and Australia. All species of *Phalaenopsis* have the same chromosome number (2n = 38) with chromosome sizes ranging from 1.5 to 3.5 μm (Arends, 1970). *Phalaenopsis* sp. can be divided into large, medium and small chromosome groups, according to their chromosome sizes. *Phalaenopsis*, occurring in the Philippines, have smaller symmetric chromosomes, while those found outside of the Philippines, have larger asymmetric chromosomes (Shindo and Kamemoto, 1963). Species of the genus *Doritis* have a very wide distribution, ranging from the Himalayas of northern India, through Thailand and Burma, to Malaysia and Indonesia (Teuscher, 1977). *Doritis pulcherrima* Lindl. is synonymous with *P. pulcherrima* (Lindl.) J. J. Smith (Christenson, 1995; Sweet, 1980) and has been used extensively in breeding with *Phalaenopsis* to produce *Doritaenopsis* such as *Doritaenopsis* Asahi obtained by Baron Toshita Iwasaki in 1923 from *D. pulcherrima* and *P. lindenii* Loher.

Interspecific hybridization and chromosome doubling are techniques often applied to produce new cultivars of orchids (Arditti, 1992). A better understanding of karyotypes and DNA contents of *Phalaenopsis* will aid in the development of new cultivars that will improve the level of production. Haploid nuclear DNA contents of angiosperms are highly variable, differing over 600-fold (Bennett and Leitch, 1995; Bennett et al., 1982). Although intraspecific variation in genome size occurs due to environmental influences, common chromosome polymorphisms, and spontaneous aberrations, DNA content per genome is fairly constant, both between cells of an individual and between different individuals of the same species (Greilhuber, 1998; Greilhuber and Obermayer, 1997). Accurate determination of genome size provides basic information for breeders and molecular geneticists. Comparisons of amounts of nuclear DNA are also useful in cytotaxonomic and evolutionary studies. The rapid technique of flow cytometry is widely used in studies of cell cycle analysis, chromosome ploidy, and nuclear DNA content in biological systems (Jones et al., 1998; Lysák et al., 1999; Ormerod, 1994). In this study we examined the nuclear DNA contents in 18 *Phalaenopsis* sp. and *D. pulcherrima* using a flow cytometer.

Endoreduplication is widespread in large numbers of higher plants (Cebolla et al., 1999; Gendreau, et al., 1999; Grafi and Larkins, 1995; Sun et al., 1999). Sturdier flowers and better forms may accompany an increase in ploidy in orchids. Although chromosome doubling is a technique for breeders to obtain new cultivars, little is known regarding the mechanism of endoreduplication in orchids. Use of flow cytometry in determining polyploidy is fast and makes determining polyploidy easier. We also demonstrate the occurrence of endoreduplication in different tissues of *P. equestris*.

Materials and Methods

Plant material. Samples of young leaves or other organs of 18 nursery bred *Phalaenopsis* sp. and *D. pulcherrima* were

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obtained from the Taiwan Sugar Research Institute (TSRI). *Phalaenopsis aphrodite* used in the experiments is native in Taiwan. Plants were maintained in a growth chamber at 14 h days/10 h nights of 26/25 °C with a 14-h photoperiod of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by fluorescent light. Day/night relative humidity was 80% /90%. Plants were watered every 2 days and fertilized monthly with Hoagland's solution.

Preparation of nuclei suspension. Suspensions of nuclei were prepared according to the protocol of Arumuganathan and Earle (1991) with some modifications. *Phalaenopsis* sp. contain large amounts of calcium oxalate crystals in their cells. The presence of crystals decreases the yield of intact nuclei. Consequently, a relatively high level of underlying debris and chemical residue that might fluoresce contaminate the samples. Therefore, a cotton filter was designed to remove the crystals. Cosmetic cotton was cut into 5 × 1-cm pieces, wetted with $MgSO_4$ buffer containing 10 mM $MgSO_4 \cdot 7H_2O$, 50 mM KCl, 5 mM Hepes (pH 8), and rolled up loosely. The cotton ball was inserted into a 5 mL pipette tip and rinsed with 6 mL $MgSO_4$ buffer to remove floating fibers. One hundred fifty milligrams of tissue from healthy plants was cut into 0.5 mm pieces using a scalpel in 3 mL of solution A in a petri dish (35 × 10 mm) on ice. Solution A contained $MgSO_4$ buffer with 6.5 mM dithiothreitol, 0.25% Triton X-100 and 0.1 $mg \cdot mL^{-1}$ propidium iodide (Calbiochem, La Jolla, Calif.). The homogenate was filtered through the cotton column into a 15 mL centrifuge tube. Four milliliters of solution A was added to the cotton filter to elute the nuclei. The eluted nuclei were centrifuged at 100 g_n for 10 min and the supernatant was discarded. The pellet was resuspended in 400 µL solution B that is solution A with 1.25 $\mu g \cdot mL^{-1}$ RNase (DNase-free) and incubated for 15 min at 37 °C. Nuclei isolated from *Pisum sativum* L. 'Minerva Maple' were used as an internal reference standard (IRS) for each sample (Johnston et al., 1999). Seeds of *P. sativum* were kindly provided by the Royal Botanic Gardens, Kew, United Kingdom. Samples were prepared from at least five plants of each clone except for *P. cornu-cervi*, *P. fasciata*, *P. gigantea*, *P. mariae*, *P. micholitzii*, *P. modesta*, and *P. parishii*, which were from leaf sections of a single plant. Each mean was obtained from at least five replications.

Flow cytometry analysis and estimation of nuclear DNA content. DNA contents of the nuclei were estimated by the relative fluorescence of samples containing a minimum of 10,000 stained nuclei per sample and were determined by using a FACSCAN flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) equipped with an argon-ion laser emitting at 488 nm. Forward scatters (FSC) and side-angle scatters (SSC) were used to exclude cellular debris. The gain of the fluorescence amplifier was adjusted so that the modes of the peaks corresponding to the nuclei fell at a desired position within the scale of the histogram of linear fluorescence intensity. The propidium iodide signals were separated with a 560-nm shortpass dichroic mirror and collected with a 585/42 bandpass (FL2).

Values for nuclear DNA content were estimated by comparison of the nuclear peak of the orchid nuclei on the linear scale with the peak for *P. sativum* nuclei included as an internal control in each run. A value of 9.56 pg/2C was used for the *P. sativum* nuclear DNA (Johnston et al., 1999). Data were analyzed using the CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) and ModFit LT software 2.0 (Verity Software House, Topsham, Maine). Histograms developed by flow cytometry were subjected to ModFit LT to detect peaks and to determine the proportion of each nuclei ploidy using a manual analysis. The quality of DNA histograms was determined by the SE of the peak, which was calculated from the SD.

Determination of chromosome number. Root tips 0.8 cm in length were severed and pretreated in 2 mM 8-hydroxyquinoline for 4 h at 18 to 20 ºC. After fixing in a 3:1 mixture of 95% ethanol and glacial acetic acid overnight, samples were washed twice with sterile distilled water and incubated in 1 M HCl at 60 ºC for 7 min. Samples were washed again and stained with 0.5% basic fuchsin for 2 h in the dark, and then treated with 1% pectinase for 40 min. Subsequently, the root tips were squashed and stained with 1% acetic orcein to determine chromosome numbers.

Results

Nuclear DNA contents of *Phalaenopsis* sp. and *Doritis pulcherrima*. Flow cytometric analysis of isolated nuclei resulted in histograms of the relative nuclear DNA content containing peaks corresponding to the G_0/G_1 nuclei of leaves of the internal reference standard *P. sativum* (Fig. 1A) and *P. aphrodite* W01-38 (Fig. 1B), W01-41 (Fig. 1C), and W01-22 (Fig. 1D) as illustrated in Fig. 1. Systematically, three peaks were found in *Phalaenopsis*, corresponding to 2C, 4C, and 8C nuclei (Fig. 1B). Genome sizes of 18 *Phalaenopsis* sp. and *D. pulcherrima* are listed in Table 1. Chromosome numbers for all the orchids examined and stained by basic fuchsin and acetic orcein were confirmed to be 2n = 2x = 38 except for the *P. aphrodite* W01-41 (2n = 3x = 57) and W01-22 (2n = 4x = 76). There was a 6.07–fold variation in genome size within 18 *Phalaenopsis* species, ranging from 2.74 pg/2C for *P. sanderiana* to 16.61 pg/2C for *P. parishii*. The genome size is small for *P. aphrodite* (2.80 pg/2C), *P. sanderiana* (2.74 pg/2C), and *P. stuartiana* (3.13 pg/2C) in section *Phalaenopsis* and *P. equestris* (3.37 pg/2C) in section *Stauroglottis*. The nuclear DNA content of section *Amboinenses* ranged from 5.28 pg/2C for *P. gigantea* to 14.50 pg/2C for *P. amboinensis* W25-08. Among the eight species in section *Zebrinae*, the variation is 2.92-fold, ranging

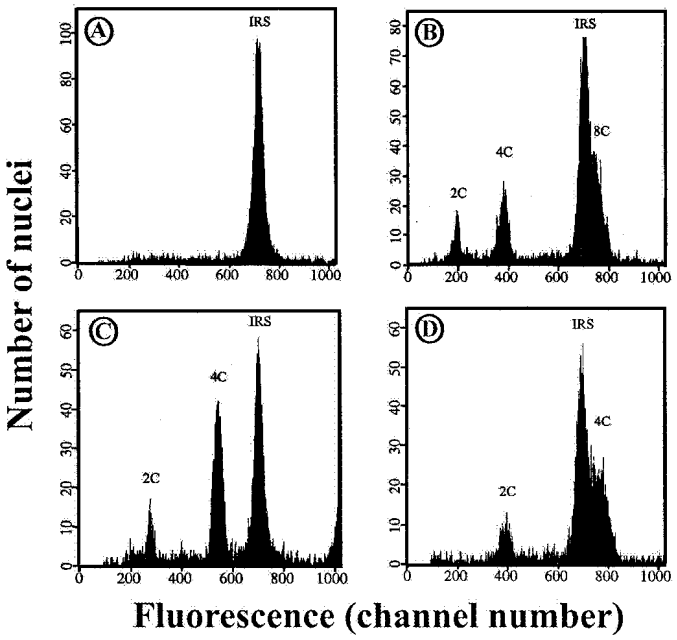

Fig. 1. Histograms of relative DNA content obtained during analysis of *P. aphrodite* W01-38, W01-41, and W01-22. Nuclei isolated from *Pisum sativum* 'Minerva Maple' were used as an internal reference standard (IRS). (**A**) *Pisum sativum*, 9.56 pg/2C DNA, (**B**) *P. aphrodite* W01-38, 2.80 pg/2C DNA, (**C**) *P. aphrodite* W01-41, 4.52 pg/2C DNA, (**D**) *P. aphrodite* W01-22, 5.89 pg/2C DNA. The 2C peak position of the orchid nuclei reflects the DNA content of each clone.

from 5.15 pg/2C for *P. modesta* to 15.03 pg/2C for *P. bellina*. In section *Polychilos*, *P. mannii* W25-07 has a genome size of 13.61 pg/2C, which is 2.11-fold of that of *P. cornu-cervi* (6.44 pg/2C). The nuclear DNA content of *D. pulcherrima* is 13.49 pg/2C, 4.92-fold that of *P. sanderiana*. Both *P. amboinensis* and *P. mannii* had two TSRI clones included in this study and exhibited almost no variation in their nuclear DNA contents. The value of nuclear DNA contents of *P. amboinensis* W02-07 (14.36 pg/2C) is close to that of W02-08 (14.50 pg/2C). Similarly, genome size of *P. mannii* W25-07 (13.50 pg/2C) is similar to that of W25-06 (13.61 pg/2C). Groups resulting from statistical analysis of data on DNA content are listed in the right column of Table 1. All of the species examined in section *Phalaenopsis* were placed in the A group, which contains the smallest genome size among the groups.

Increases in nuclear DNA content due to increases in chromosome number in *P. aphrodite*. The three TSRI cultivars *P. aphrodite*, W01-38, W01-41, and W01-22, contained nuclear DNA contents of 2.80, 4.52, and 5.89 pg/2C, respectively (Table 2). Each mean was obtained from at least six replications prepared from different plants of the clone. The ratio of nuclear DNA contents of these three TSRI clones was 1:1.6:2.1. Fuchsin staining indicated that the somatic chromosome numbers of the W01-38, W01-41, and W01-22 clones were 38, 57, and 76, respectively (Fig. 2). Nuclear DNA content versus ploidy for these three clones was analyzed using linear regression and resulted in an *r* value of 0.998. Apparently, the nuclear DNA contents of these *P. aphrodite* clones have a linear relationship with their ploidy levels.

Endoreduplication occurring in different tissues of *P. equestris*. Leaves of pea contained mainly 2C nuclei (>80%), however, endoreduplicated nuclei (≥4C) were detected in all the *Phalaenopsis* sp. examined. Therefore, the levels of 2C, 4C, 8C, 16C, and 32C nuclei in various tissues of *P. equestris* were investigated. Apparently, nuclei isolated from leaves, flowers, and root tips have different degrees of endoreduplication. Table 3 shows that *P. equestris* contained different levels of endoreduplicated nuclei with 4C, 8C, and 16C DNA contents in leaves, flowers, and root tips. Flowers contained 4C nuclei (62.4%) more than 8C nuclei (20.3%). Similarly, root tips also contained 4C nuclei (41.1%) more than 8C nuclei (25.2%). However, leaf tissues contained a high level of 8C nuclei (54.6%), but low levels of 2C (4.0%) and 4C nuclei (27.7%), as compared to flowers or root tips. The content of 8C and 16C nuclei was 68.3% in leaves, 35.9% in root tips, and 23.3% in flowers. There were four replications in this experiment. Each mean was obtained from four different plants. The original data were treated as 4-variate Gaussian observations and analyzed with multivariate analysis of variance. Our results indicated that the levels of endoreduplication of nuclei isolated from leaves, flowers, and roots were significantly different at $P = 0.01$.

Discussion

Elimination of crystals in leaf samples. Endogenous factors such as developmental stages and portions of tissues or organs used, affect the yield of nuclei. Our results indicated that young leaf tissue yielded more nuclei than old leaf tissue, thus only young leaves were used for DNA content determination as illustrated in Table 1. Debris of broken nuclei, chloroplasts, mitochondria, and other cytoplasmic particles produced

Table 1. Nuclear DNA contents and groups based on LSD ($P = 0.05$) of 18 *Phalaenopsis* sp. and *Doritis pulcherrima* determined by flow cytometry of propidium iodide stained nuclei using *Pisum sativum* 'Minerva Maple' as a standard.

Genus	Section[z]	Species[z]	TSRI clone[y, x]	Mean (pg/2C)	SE	N	Group
Phalaenopsis	*Phalaenopsis*	*P. aphrodite* Rchb.f.	W01-38	2.80	0.06	6	A
		P. sanderiana Rchb.f.	W36-01	2.74	0.04	5	A
		P. stuartiana Rchb.f.	W40-04	3.13	0.07	11	A
	Stauroglottis	*P. equestris* (Schauer) Rchb.f.	W09-51	3.37	0.05	16	A
	Amboinenses	*P. amboinensis* J. J. Smith	W02-07	14.36	0.19	15	F
		P. amboinensis J. J. Smith	W02-08	14.50	0.23	12	F
		P. gigantea J. J. Smith	S82-314	5.28	0.07	5	B
		P. micholitzii Rolfe	W27-05	6.49	0.13	5	C
		P. venosa Shim & Fowl.	W42-01	9.52	0.27	12	D
	Zebrinae	*P. fasciata* Rchb.f.	W10-01	6.56	0.07	5	C
		P. lueddemanniana Rchb.f.	W23-02	6.49	0.22	5	C
		P. modesta J. J. Smith	W28-06	5.15	0.24	5	B
		P. mariae Burb. ex Warn. & Wms.	W26	6.48	0.11	5	C
		P. pulchra (Rchb.f.) Sweet	W33-04	6.37	0.22	5	C
		P. sumatrana Korth. & Rchb.f.	W41-03	6.62	0.06	7	C
		P. bellina (Rchb.f.) Cristenson[w]	S82-429	15.03	0.21	15	G
	Polychilos	*P. cornu-cervi* (Breda) Bl & Rchb.f.	W08-01	6.44	0.16	5	C
		P. mannii Rchb.f.	W25-06	13.50	0.12	5	E
		P. mannii Rchb.f.	W25-07	13.61	0.19	5	E
	Parishianae	*P. parishii* Rchb.f.	W32	16.61	0.29	5	H
Doritis[v]		*D. pulcherrima* Lindl.	W46-21	13.49	0.21	18	E

[z]Classification according to Sweet (1980).
[y]Source of plant material: Taiwan Sugar Research Institute (TSRI).
[x]Chromosome numbers are 2n = 2x = 38.
[w]Nomenclature according to Christenson and Whitten (1995).
[v]Classification according to Teuscher (1977).

Table 2. Nuclear DNA contents of *P. aphrodite* W01-38, W01-41, and W01-22 clones determined by flow cytometry of propidium iodide stained nuclei using *P. sativum* as a standard. Chromosome numbers observed by basic fuchsin and acetic orcein staining are illustrated in Fig. 2.

TSRI clone	Chromosome no.	Mean DNA content (pg/2C)	SE	N
W01-38	38	2.80	0.06	6
W01-41	57	4.52	0.16	7
W01-22	76	5.89	0.10	10

fluorescences that can be eliminated by choosing a gate region. In the case of *Phalaenopsis* and *Doritis*, calcium oxalate crystals exist in plant tissues, especially leaves. These crystals tended to clog the tubing inside the flow cytometer and block the flow stream. The cotton filter developed in this research successfully removed the calcium oxalate crystals and may prove useful for other species containing similar crystals.

The relationship between nuclear DNA contents and chromosome numbers in *P. aphrodite*. The nuclear DNA contents of *P. aphrodite* W01-38, W01-41, and W01-22 had a ratio near 2:3:4, corresponding to their somatic chromosome numbers, 38, 57, and 76 (Table 2 and Fig. 2). The linear relationship between nuclear DNA contents and chromosome numbers of these three clones indicated the accuracy of flow cytometry.

The relationship between 2C genome values and chromosome sizes. This study presents nuclear DNA contents, as determined by flow cytometry of 18 *Phalaenopsis* sp. and *D. pulcherrima* (Table 1). Our data for the nuclear DNA content of *P. aphrodite* W01-38 (2n = 38), 2.80 pg/2C, is similar to a previously reported value of 2.34 pg/2C determined by reassociation kinetics (Capesius and Nagl, 1978). *Phalaenopsis aphrodite*, *P. sanderiana*, *P. equestris*, and *P. stuartiana* were placed in the same A group based on LSD. Interestingly, the *P. aphrodite*, *P. equestris*, and *P. stuartiana* that had lower DNA contents were similar phylogenetically as analyzed by randomly amplified polymorphic DNA markers (Fu et al., 1997). Data herein support the grouping of *P. aphrodite*, *P. sanderiana*, and *P. stuartiana* into section *Phalaenopsis* (Sweet, 1980) and suggest that *P. equestris* is closely related to section *Phalaenopsis*. A repetitive DNA (*Pvr*I) was found in genomic DNA of the large genome size species *P. vensosa* (9.52 pg/2C), *P. amboinensis* (14.36 pg/2C), *P. bellina* (15.03 pg/2C), and *D. pulcherrima* (13.49 pg/2C) but not in that of the small or medium genome size species *P. aphrodite* (2.80 pg/2C), *P. equestris* (3.37 pg/2C), and *P. lueddemanniana* (6.49 pg/2C), as revealed by Southern analysis and fluorescence in situ hybridization (C. C. Chen, unpublished data). The latter are species native to the Philippines. The former are extra-Philippine species with chromosomes being two to three times larger than those of the Philippine species (Shindo and Kamemoto, 1963). In *Lathyrus* sp. the quantitative DNA variation associated with speciation was mainly attributable to variation in the repetitive component as estimated by C_0t reassociation of the DNA (Narayan and Rees, 1976). Perhaps the *Phalaenopsis* sp. with high nuclear DNA contents also comprised more repetitive DNA in their genomes. Genome size for *P. sanderiana* is the smallest among the orchids tested. Karyotype analysis revealed that *P. lueddemanniana* contained medium size chromosomes and *P. mannii* and *P. bellina* possessed large size chromosomes (Shindo and Kamemoto, 1963). We found the genome sizes of these four species to be 2.74, 6.49, 13.50, and 15.03 pg/2C, respectively. Therefore, we postulate that genome sizes of *Phalaenopsis* sp. are proportional to their chromosome sizes. Integration of molecular, phylogenetic and cytogenetic analysis of repetitive DNA may shed light on evolution of *Phalaenopsis*.

Intraspecific variation in genome size. No variation in DNA content was found between the two different *P. amboinensis* clones, W02-07 (14.36 pg/2C) and W02-08 (14.50 pg/2C); or between the two different *P. mannii* clones, W25-07 (13.50 pg/2C) and W25-06 (13.61 pg/2C) (Table 1). Mean DNA content of the large chromosome species *D. pulcherrima* W46-21 was calculated to be 13.49 pg/2C, which is larger than a previously reported value of 9.25 pg/2C (Jones et al., 1998). Moreover, our data of DNA 2C-values of 3.37 pg/2C for *P. equestris* and 6.49 pg/2C for *P. lueddemanniana* are smaller than those of their results (5.53 pg/2C for *P. equestris* and 8.65 pg/2C for *P. lueddemanniana*). These differences could have resulted from

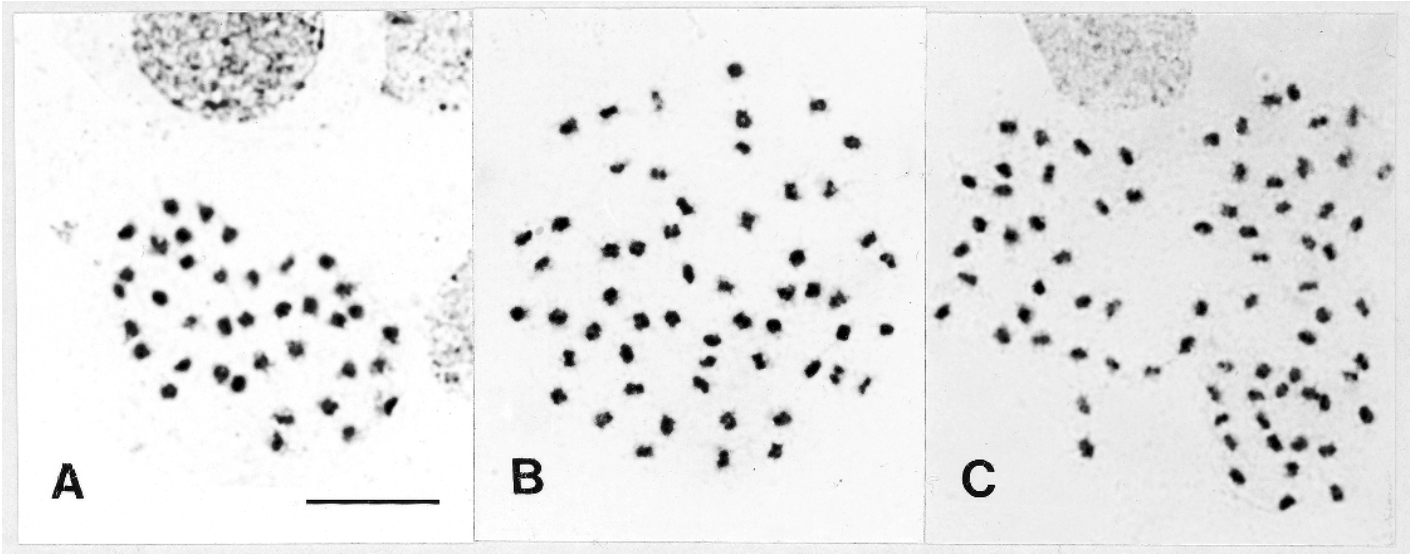

Fig. 2. Mitotic metaphase of clones (**A**) W01-38, (**B**) W01-41, and (**C**) W01-22 of *P. aphrodite* stained by basic fuchsin showing 38, 57, and 76 chromosomes, respectively. Bar = 10 µm.

Table 3. Ploidy patterns of *P. equestris* in different tissues.

		Ploidy patterns (% distribution of nuclei)				
Tissue		2C	4C	8C	16C	32C
Flower	Mean	14.3	62.4	20.3	3.0	0.0
	SE	1.7	0.7	1.5	0.9	0.0
Root	Mean	20.3	41.1	25.2	10.8	2.7
	SE	2.8	0.9	0.9	1.7	0.5
Leaf	Mean	4.0	27.7	54.6	13.7	0.0
	SE	0.6	2.5	2.1	1.0	0.0

intraspecific variation. Alternatively, the variation could be due to the difference in the internal reference standard, which is *P. sativum* in our experiments and red blood cells of chicken in the previous study (Jones et al., 1998). We compared the histogram of a sample with the internal standard to that of the sample alone to obtain a precise 2C peak.

Endoreduplication occuring in different tissues of *P. equestris*. Results herein demonstrate the presence of endoreduplication in *Phalaenopsis*, similar to that observed in *Dendrobium* Sw. sp. and cultivars (Jones and Kuehnle, 1998). Different levels of endoreduplication were detected in various tissues of *P. equestris* (Table 3). Obviously, leaves contained more nuclei with polyploidy of 8C and 16C than flowers and roots. *Phalaenopsis* may have become endoreduplicated by repeated rounds of replication of their entire genomes in the absence of mitosis. The highest degree of endoreduplication was detected in *Phalaenopsis* leaves.

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