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FLOW CYTOMETRY ANALYSIS REVEALS NUCLEAR DNA CONTENT VARIATION IN *PHALAENOPSIS* YOUNG LEAF AND ROOT TIP CELLS

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SUMMARY

Phalaenopsis is one of the most popular orchid genera, exhibiting a variety of flower morphologies. Ploidy level was a factor influencing the success of *Phalaenopsis* crossbreeding. This research aimed to determine the ploidy level by flow cytometry and correlation with morphological characteristics on six hybrid cultivars of *Phalaenopsis* (1702, 1819, 1981, 3903, 3904, and 3908). Flow cytometry analysis on young leaves and root tip cells was also carried out on *P. amabilis*, *P. amboinensis*, and *P. schilleriana.* Research results showed multiple peaks of fluorescence appeared on the flow cytometry histogram. The young leaves and root tip cells show the distribution of cells into different ploidy levels and indicate mixoploid and endopolyploid conditions of the cells in both leaves and root samples. This complexity makes it challenging to determine the ploidy level of each cultivar and the correlation between morphological characteristics and ploidy level, even though the weak positive correlation was prominent between flower numbers and genome size. These findings serve as foundational data on the cytogenetic background of the cultivars for orchid breeders in crossbreeding, selection of haploid plants, mutation analysis, and variety consistency monitoring.

Keywords: *Phalaenopsis*, DNA contents, flow cytometry, genome size, mixoploid, ploidy level, morphological characterization

Key findings: Flow cytometry analysis revealed a mixoploid condition; however, the ploidy level of hybrid cultivars and species may be triploid or tetraploid. A weak positive correlation between flower number and genome size was apparent, but no definitive morphological indicators for ploidy levels emerged.

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INTRODUCTION

Orchid (*Orchidaceae*) represents one of the extensive families of flowering plants (Willis, 2017), comprising approximately 763 genera and over 28000 species (Chase *et al*., 2015; Govaerts *et al*., 2017; Tsai *et al*., 2017). Among these, specific genera have gained prominence in commercial production and marketing, including *Dendrobium, Phalaenopsis, Vanda, Paphiopedilum, Bulbophyllum, Cattleya, Cymbidium, Ascocenda, Brassia, Miltonia,* and *Epidendrum* (De and Pathak, 2015). Of these genera, *Phalaenopsis* stands out as one of the most popular and economically valuable genera owing to its high demand as an ornamental plant (Hinsley *et al.,* 2018; Li *et al.,* 2021). With approximately 70 species (Cribb and Schuiteman, 2012), *Phalaenopsis* has been subjected to intensive breeding efforts over the years, resulting in a diverse array of hybrid varieties (Royal Society of Horticulture, 2018). These hybrid varieties, characterized by long-lasting flowers that can endure for 3–4 months, play a significant role in the orchid market (United States Department of Agriculture, 2018).

The advanced *Phalaenopsis* hybrid cultivars comprised three main groups: standard broad flower cultivars with mono-color petals (white, purple, or yellow), Harlequin (novelty) cultivars featuring diverse and intense flower color with blotches, and multiflora cultivars with smaller plant sizes and multiple branching inflorescences, often with a higher flower number.

Interestingly, some of the superior *Phalaenopsis* hybrid varieties, such as "Big White Flower" and hybrids with yellow or red coloration (*P. Sunrise "Goldamour," P. Taipei Gold, Dpts*. Sinica Sunday*,* and *Dpts*. Sweet Strawberry "Wei") had shown as polyploids (Chen *et al.,* 2011). Polyploidy, characterized by an increase in the number of chromosome sets, correlates with desirable traits, such as larger flower size, intense coloring, and aromatic fragrance than diploid plants (Sattler *et al*., 2016; Vilcherrez-Atoche *et al*., 2022). Harnessing these traits by breeding polyploid varieties has become a key focus in *Phalaenopsis* breeding programs, offering the potential for accelerated development of new varieties. However, breeding efforts involving complex hybrid varieties are sometimes hampered by pod abortion or the production of seedless pods (Sukma *et al*., 2015). Therefore, determining the ploidy level of parental plants before crossing is essential to improving breeding programs' success rate and efficiency.

Phalaenopsis species are mostly diploid with the chromosome number 2n=2x=38. However, most of the standard *Phalaenopsis* hybrid cultivars are tetraploid with the chromosome number 2n=4x=76 (Lee *et al*., 2020). According to Chen and Tang (2017), some plant species with similar ploidy levels differ in genome sizes. For example, flow cytometry analysis conducted on 50 species of *Phalaenopsis* revealed a high variation of genome size (DNA contents) in the range of 2.77 pg in *P. philippinensis* to 17.47 pg in *P. lobii* (Chen and Tang, 2017), while based on Lee *et al.* (2017), they varied among *Phalaenopsis* species (from 1C = 1.39–8.74 pg). DNA content has a positive correlation with the total complement length of a chromosome, indicating a positive correlation with genome size (Chen and Tang, 2017). However, the determination of the ploidy level of the hybrid cultivars based on DNA contents is challenging due to differences in genome introgression and the diverse genome sizes of the parental species used for hybrid cultivars (Kron, 2015; Escobedo-Garcia-Medrano *et al*., 2018; Lee *et al*., 2020). Moreover, no information about morphological traits exists to indicate *Phalaenopsis* ploidy level, demonstrating a positive correlation with the cultivar ploidy level. Therefore, the cytogenetic background of cultivars requires elucidating before their application in breeding programs.

While determining plant ploidy level through chromosome counting at the metaphase stage using a microscope is widely practiced, this method requires highly skilled personnel and is time-consuming (Tomaszewska *et al*., 2021). Flow cytometry, utilizing fluorescently labeled nuclei suspensions, is a convenient, rapid, and reproducible technique for ploidy determination (Kron, 2015; Escobedo-Garcia-Medrano *et al*.,

2018). In this study, we characterized and determined the DNA content of the three species and six commercial *Phalaenopsis* cultivars using flow cytometry to explore any relationships between morphological traits and DNA content to predict ploidy levels virtually.

MATERIALS AND METHODS

The study area

The morphological characterization of orchids commenced at the Laboratory of Plant Molecular and Biology, Department of Agronomy and Horticulture, Faculty of Agriculture, IPB University, Bogor, Indonesia. Meanwhile, ploidy analysis proceeded at the Laboratory of Cell and Tissue Biology, Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Cimanggu, Bogor, Indonesia. The study spanned August to November 2022.

Procedures

Plant material

Three mature *Phalaenopsis* plant species, *P. amabilis* (PAB), *P. amboinensis* (PAM), and *P. schilleriana* (PSC), came from our collection. Meanwhile, the PT Wilis Agro Lestari provided three commercial standard cultivars of *Phalaenopsis* hybrid (1702, 1819, 1981) and three novelty multiflora cultivars (3903, 3904, and 3908) used in this experiment (Figure 1). Morphological characterization ran on six *Phalaenopsis* hybrid cultivars, while flow cytometry analysis transpired on all three species and six cultivars.

Morphological characterization

Six commercial cultivars at the flowering stage were trials for morphological characterization based on the guidelines set by the International Union for the Protection of New Varieties of Plants (UPOV) (2003) and the Orchid Characterization of Plant Variety

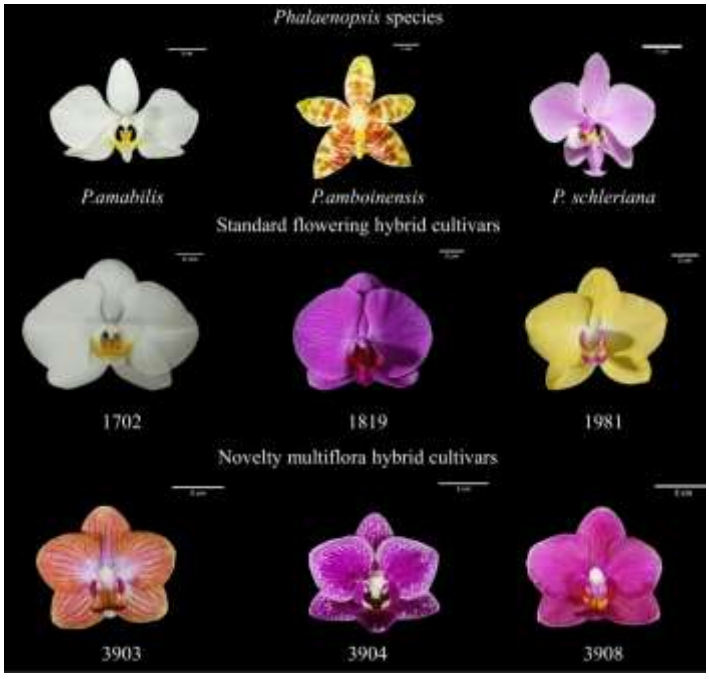

Figure 1. Flower performance of the species and hybrid cultivars in the experiment.

Protection Office, Ministry of Agriculture, Indonesia. The plant characteristics observed were plant height, stem diameter, leaf length and width, length of inflorescence, number of flowers, front flower length and width, sepal length and width, petal length and width, apical lip length and width, and anther (pollinia) length and width.

DNA nuclear content analysis by flow cytometry

The plant material utilized for the flow cytometric analysis consisted of tips from young leaves and roots, 0.5 mm × 0.5 mm in size (200 mg of fresh weight). Upon collection from the plants, the samples remained in a cool box until DNA extraction. Subsequently, gently washing the samples continued to chop finely on a cooled Petri dish in 500 µl of extraction buffer (Dolezel *et al*., 2007). The resulting suspension underwent filtering through a 50 µm nylon mesh, followed by staining with a 150 µl solution of propidium iodide (PI) and RNAse, then mixed using a vortex mixer. The nuclear cell suspension proceeded cool-incubation for 15 min in a dark room before its analysis using the Guava EasyCyte™ Flow Cytometer (Millipore Corporation, U.S.A) equipped with a laser emission at 488 nm. DNA content estimation of the nuclei depended on the relative fluorescence of samples containing a minimum of 10,000 stained nuclei per sample. Three biological replicates represented each species or cultivar. The diploid maize with a 2C content of 2,178.2361 pg served as the standard.

Data analysis

Morphological characters received analysis of variance (ANOVA) at the α level of 5%. Further analysis used the least significant difference test ($LSD_{0.05}$) if the cultivars significantly affected the plant traits. Data processing used SAS Studio software.

Histograms consisting of peaks can visualize the distribution of cells within the analyzed sample into different ploidy levels. Researchers looked at where the peaks of relative fluorescence position on the x-axis to interpret it. This determination ensued by comparing the position of peaks in the Internal Reference Standard (IRS) histogram, such as *Zea mays* (diploid), with the analyzed sample. If the sample's single peak position matches the IRS, it receives a diploid classification. If the sample's peak position is twice that of the IRS, its label is a tetraploid, four times is an octaploid, and so forth. If the sample has multiple peaks, it becomes a mixoploid or undergoes endopolyploidy.

The data and histogram results came from the flow cytometry equipment, with manual analysis carried out using Microsoft Excel. The quality of the analysis assessment relied on the proportion of background debris, aiming for minimal levels. Evaluation criteria included checking the symmetry of the peaks and assessing the fluorescence intensity distribution, quantified as the coefficient of variation (CV). A CV value below 3% is valid as good, while a CV of more than 8% is unacceptable. The CV calculation from the histogram data followed the formula according to Dolezel and Bartos (2007).

$$\text{CV}\ (\%) = \frac{\textit{Standard deviation of the peak}}{\textit{Mean channel position of the peak}} \mathbf{x\ 100}$$

DNA contents and genome size analysis continued from the flow cytometry results. We used DNA nuclear content from *Zea mays* as an Internal Reference Standard (IRS) to calculate the DNA contents (DNA content is 2,178.2361 picogram [pg] and genome size is 5.67 megabasepair [Mbp]) (Figure 2). The formula to count 2C DNA contents according to Dolezel and Bartos (2007) follows below:

$$\textit{Sample 2C DNA content} = \frac{\textit{Sample G1 peak mean}}{\textit{Standard G1 peak mean}} \text{ x standard 2C DNA content (pg)}$$

Genome size (Mbp) calculation as DNA contents in 2C nuclear in each sample had the standard of 1 pg DNA containing 980 Mbp (Dolezel *et al*., 2007; Bennett *et al*., 2000). Genome size in 2C nuclear cell computation was by multiplying the total DNA content of the sample (pg) with 980 Mbp. Pearson correlation

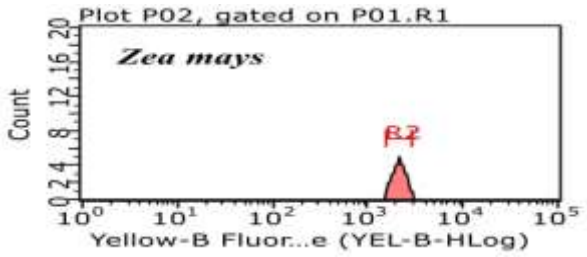

Figure 2. Histograms of *Zea mays* used as an Internal Reference Standard (IRS) to calculate the DNA content of samples.

Table 1. Plant height, stem diameter, leaf length, and leaf width of six *Phalaenopsis* hybrid cultivars.

Characteristics	Cultivars						
	Standard hybrid		Novelty multiflora				
	1702	1819	1981	3908	3904	3903	Pr (>F)
Plant height (cm)	105.33^{b}	111.33^{a}	67.40^{c}	24.37^{f}	33.20^{e}	38.20^{d}	<.0001
Stem diameter (mm)	6.50^{a}	6.23^{a}	4.87^{b}	3.20^{d}	4.07^{c}	4.30^{bc}	<.0001
Leaf length (cm)	25.70^{b}	29.70^{a}	20.67^{c}	14.70^{d}	14.60^{d}	14.43^{d}	<.0001
Leaf width (cm)	7.47^{b}	8.33^{a}	7.67^{ab}	5.60^{c}	5.40^{c}	7.23^{b}	<.0001

Note: Means in the column followed by different letters are significantly different (One-Way ANOVA, $P < 0.05$; LSD Test).

application saw the possible relationship between morphological characters and DNA nuclear contents and the prediction of the ploidy level of the plants.

RESULTS

Morphological characteristics of *Phalaenopsis* cultivars

Morphological character analysis showed significant differences between standard and novelty multiflora hybrid cultivars by giving a P–value (>F) of <0.0001 (Table 1). The standard hybrid cultivar 1819 exhibited the tallest plants, followed by the cultivar 1702. Cultivar 1981 had a medium plant height, while cultivars 3908, 3904, and 3903 were short, approximately one-third of the height of the other cultivars. The stem diameter did not significantly differ between standard hybrid cultivars 1702 and 1819; however, both have a remarkably wider stem diameter than those in the novelty multiflora hybrid cultivars (3908, 3904, and 3903).

The cultivars also significantly affected leaf length and width. Standard hybrid cultivars generally had longer leaves than the novelty multiflora hybrid cultivars. Among standard hybrid cultivars, prominent differences were visible in leaf length, with cultivar 1819 having a massive leaf. In contrast, the novelty multiflora hybrids exhibited similar leaf lengths, but cultivar 3903 had the widest leaf. Generally, based on plant height, leaf length, and width, cultivar 1819 displayed the largest plant size compared with the others.

Regarding flower characteristics (Table 2), significant differences occurred among cultivars. Cultivar 1819 had the longest inflorescence, followed by cultivars 1702, 1981, 3903, 3904, and 3908. Conversely, novelty multiflora cultivars exhibited notably more flower count, with cultivar 3903 having the highest number of flowers, followed by cultivars 3904, 3908, 1819, 1702, and 1981. Moreover, flower size and ornamentation, including front flower length and width, sepal and petal length and width, lip length and width, and anther size, were superior in standard hybrid cultivars than the novelty multiflora cultivars.

Table 2. Quantitative flower characteristics of six *Phalaenopsis* hybrid cultivars.

Characteristics	Cultivars						
	Standard hybrid			Novelty multiflora			
	1702	1819	1981	3908	3904	3903	Pr (>F)
Length of inflorescence (cm)	46.67^{b}	52.17^{a}	27.10^{c}	11.10^{e}	11.20^{e}	20.50^{d}	<.0001
Number of flowers	12.67^{c}	13.00^{c}	12.33^{c}	21.33^{b}	23.33^{b}	30.00^{a}	<.0001
Front flower length (cm)	11.37^{a}	10.03^{b}	9.47^{b}	4.67^{cd}	5.10^{c}	4.37^{d}	<.0001
Front flower width (cm)	12.50^{a}	11.40^{b}	10.03^{c}	4.13^{e}	4.73^{d}	4.33^{de}	<.0001
Sepal length (cm)	6.03^{a}	5.53^{b}	5.00^{c}	2.27^{d}	2.40^{d}	2.27^{d}	<.0001
Sepal width (cm)	4.37^{b}	4.80^{a}	3.40^{c}	1.80^{e}	1.70^{f}	2.03^{d}	<.0001
Petal length (cm)	6.10^{a}	5.37^{b}	5.53^{b}	2.17^{c}	2.33^{c}	2.17^{c}	<.0001
Petal width (cm)	7.53^{a}	7.10^{b}	4.60^{c}	2.27^{d}	2.53^{d}	2.47^{d}	<.0001
Apical lip length (cm)	2.47^{a}	2.40^{a}	2.27^{b}	1.63^{d}	1.77^{c}	1.37^{e}	<.0001
Apical lip width (cm)	2.87^{a}	2.63^{b}	2.23^{c}	1.37^{e}	1.70^{d}	1.43^{e}	<.0001
Anther length (mm)	0.33^{a}	0.30^{ab}	0.27^{b}	0.17^{c}	0.20^{c}	0.17^{c}	<.0001
Anther width (mm)	0.20^{a}	0.20^{a}	0.17^{b}	0.15^{bc}	0.10^{d}	0.14^{c}	<.0001

Note: Means in the column followed by different letters are significantly different (One-Way ANOVA, $P < 0.05$; LSD Test).

Nuclear DNA variations of *Phalaenopsis* cultivars

The results of Flow Cytometry (FCM) histogram analysis showed a single peak for the diploid *Zea mays* (Figure 2) as opposed to four main peaks for the *Phalaenopsis* cultivars (Figures 3 and 4), which meant the occurrence of endopolyploidy in the analyzed samples. The assessed young leaf cells and root tips indicated the presence of certain endopolyploidy with a maximum ploidy pattern of 16C (peak R5). Peaks refer to data distribution patterns that appear in a histogram. Peaks indicate groups of cells that have similar properties or characteristics in the probed samples.

The core distribution of each peak is called the ploidy pattern, which has different levels marked by C values (2C, 4C, 8C, and 16C); thus, the designation is R2 = 2C; R3 = 4C; R4 = 8C; R5 = 16C. Figure 3 illustrates the diverse DNA contents observed in the young leaf tip cells. Three chief peaks labeled R2, R3, R4, and R5 emerged in leaf cells. Among the cultivars, 1702, 1819, 3903, 3908, PAB, and PSC predominantly contained cells at the R4 level (8C), while cultivars 1981, 3904, and PAM mainly exhibited cells at the R3 level (4C). In contrast to leaf cells, which have the highest nuclear distribution at the R3 level (4C) and R4 level (8C), Figure 4 shows the root tip cells of the cultivars, with R3 (4C) as the predominant peak.

Table 3 presents relative inflorescence, CV, DNA contents, and 1C genome size from young leaf cells. The 2C DNA contents, arranged from highest to lowest, are on cultivars 3908 (15.42 pg), 3903 (11.76 pg), 3904 (10.89 pg), 1981 (10.26 pg), 1819 (8.95 pg), 1702 (5.66 pg), PAB (4.74 pg), PSC (3.94 pg), and PAM (3.69 pg). In general, hybrid varieties exhibited higher 2C DNA contents than the species. Interestingly, the genome size also follows this trend.

Table 4 shows the DNA content calculation and genome size from root samples. CV values appeared to be higher in leaf cells than in root cells, as shown in Table 3. Ranking cultivars depended on their 2C DNA contents, with 3908 having the highest content, followed by PAM (15.56 pg), 3908 (13.75 pg), 3903 (11.42 pg), 1981 (11.41 pg), 3904 (9.39 pg), 1819 (8.81 pg), 1702 (8.10 pg), PAB (5.35 pg), and PSC (4.67 pg). Generally, trends in 2C DNA contents from leaf cells are similar to those from root cells, except for PAM, which exhibited a deviation. The presence of multiple peaks of inflorescence on the flow cytometry histogram indicates a mixoploid condition of the cells in both leaves and root samples. This complexity makes it

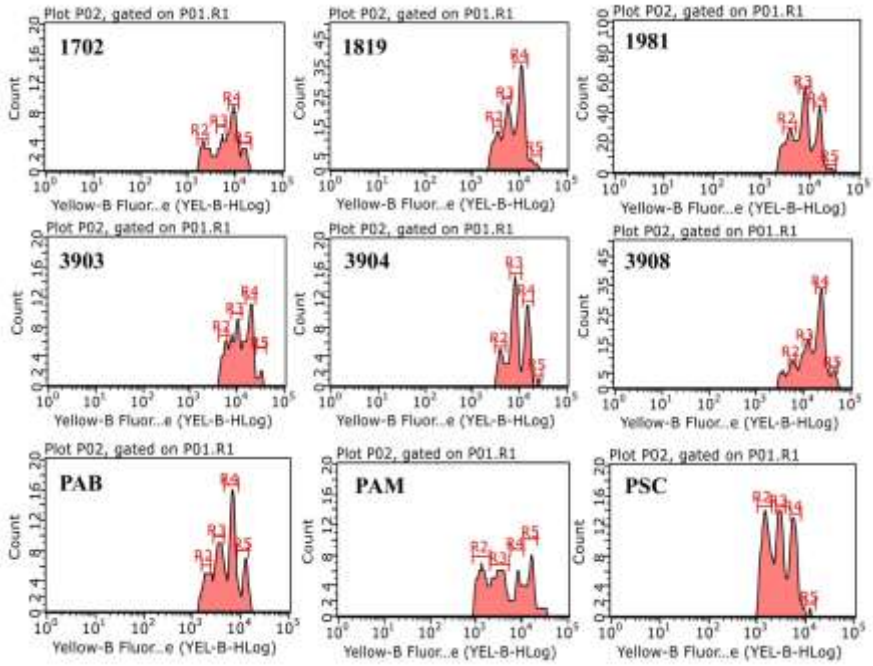

Figure 3. Histograms of relative fluorescence intensities showing ploidy levels were obtained after analysis of nuclei isolated from young leaf tissues of *Phalaenopsis* hybrid cultivars (1702, 1819, 1981, 3903, 3904, and 3908) and *Phalaenopsis* species (*P. amabilis* [PAB], *P. amboinensis* [PAM], and *P. schilleriana* [PSC]).

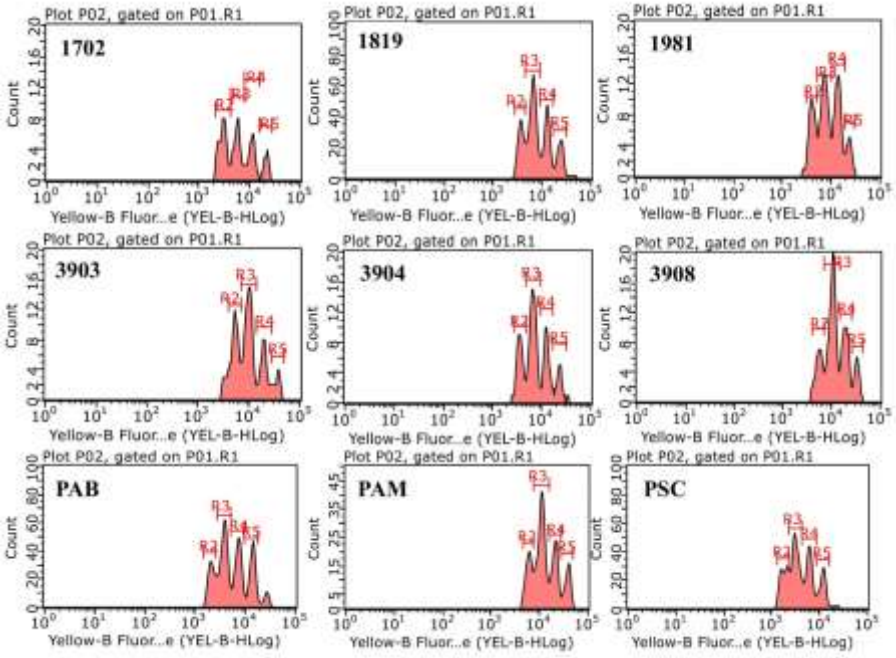

Figure 4. Histograms of relative fluorescence intensities showing ploidy levels were obtained after analysis of nuclei isolated from young root tissues of *Phalaenopsis* hybrid cultivars (1702, 1819, 1981, 3903, 3904, and 3908) and *Phalaenopsis* species (*P. amabilis* [PAB], *P. amboinensis* [PAM], and *P. schilleriana* [PSC]).

Table 3. Estimation of DNA contents and genome size from leaf samples of *Phalaenopsis* hybrid cultivars and species.

Species/ Cultivars	Peaks	Relative fluorescence (Mean ± SD)			CV (CV (%)	2C DNA Content (pg)	1C Genome size (Mbp)	Estimated Ploidy
1702	R2	2,174.00	±	30.80	1.42	5.66	2,773.30	2n=2C
	R3	4,789.00	±	180.40	3.77	12.47	6,109.49	2n=4C
	R4	8,698.00	±	632.30	7.27	22.64	11,094.86	2n=8C
	R5	15,750.00	±	607.10	3.85	41.00	20,089.63	2n=16C
1819	R2	3,437.00	±	41.90	1.22	8.95	4,384.74	2n=2C
	R3	5,805.00	±	392.80	6.77	15.11	7,405.10	2n=3C
	R4	10,629.00	±	1,680.30	15.81	27.67	13,557.13	2n=6C
	R5	19,811.00	±	2,850.30	14.39	51.57	25,269.77	2n=12C
1981	R2	3,943.00	±	221.93	5.63	10.26	5,029.30	2n=2C
	R3	7,928.00	±	13.53	0.17	20.64	10,113.17	2n=4C
	R4	14,948.00	±	643.74	4.31	38.91	19,066.26	2n=8C
	R5	26,917.00	±	2,125.58	7.90	70.07	34,332.66	2n=16C
3903	R2	4,519.00	±	95.06	2.10	11.76	5,764.54	2n=2C
	R3	9,099.00	±	352.81	3.88	23.69	11,606.41	2n=4C
	R4	16,658.00	±	1,003.63	6.02	43.36	21,247.09	2n=8C
	R5	31,026.00	±	611.79	1.97	80.76	39,573.30	2n=16C
3904	R2	4,184.00	±	205.77	4.92	10.89	5,337.55	2n=2C
	R3	8,408.00	±	503.05	5.98	21.89	10,725.30	2n=4C
	R4	14,693.00	±	519.78	3.54	38.25	18,741.70	2n=8C
	R5	24,463.00	±	2,557.77	10.46	63.68	31,203.26	2n=16C
3908	R2	5,924.00	±	51.12	0.86	15.42	7,556.23	2n=2C
	R3	12,277.00	±	215.66	1.76	31.96	15,660.06	2n=4C
	R4	23,550.00	±	505.79	2.15	61.30	30,038.30	2n=8C
	R5	42,361.00	±	779.67	1.84	110.27	54,030.85	2n=16C
PAB	R2	1,820.00	±	199.58	10.96	4.74	2,322.13	2n=2C
	R3	3,120.00	±	558.49	17.90	8.12	3,980.41	2n=4C
	R4	5,646.00	±	918.54	16.27	14.70	7,201.98	2n=6C
	R5	10,799.00	±	1,316.56	12.19	28.11	13,774.68	2n=12C
PAM	R2	1,418.00	±	46.41	3.27	3.69	1,809.81	2n=2C
	R3	3,363.00	±	140.84	4.19	8.75	4,289.76	2n=4C
	R4	7,530.00	±	830.41	11.03	19.60	9,605.07	2n=10C
	R5	14,731.00	±	8,564.87	58.14	38.35	18,789.52	2n=20C
PSC	R2	1,512.00	±	171.35	11.33	3.94	1,928.94	2n=2C
	R3	3,190.00	±	241.16	7.56	8.30	4,068.95	2n=4C
	R4	6,063.00	±	122.48	2.02	15.78	7,734.00	2n=8C
	R5	11,513.00	±	460.98	4.00	29.97	14,684.92	2n=16C

Phalaenopsis hybrid cultivars: 1702, 1819, 1981, 3903, 3904, 3908; PAB: *P. amabilis*; PAM: *P. amboinensis*; PSC: *P. schilleriana*; SD: Standard Deviation; CV: Coefficient of Variance.

Table 4. Estimation of DNA contents and genome size from root samples of *Phalaenopsis* hybrid cultivars and species.

Genotype	Peaks	Relative fluorescence (Mean ± SD)			CV (%)	2C DNA Content (pg)	1C Genome size (Mbp)	Estimated Ploidy
1702	R2	3,111.00	±	145.30	4.67	8.10	3,968.85	2n=2C
	R3	5,724.00	±	188.30	3.29	14.90	7,301.23	2n=3C
	R4	10,874.00	±	562.90	5.18	28.31	13,870.27	2n=6C
	R5	21,083.00	±	918.80	4.36	54.88	26,892.16	2n=14C
1819	R2	3,384.00	±	414.30	12.24	8.81	4,317.28	2n=2C
	R3	6,671.00	±	185.60	2.78	17.37	8,509.17	2n=3C
	R4	12,847.00	±	327.70	2.55	33.44	16,386.53	2n=8C
	R5	23,195.00	±	1,585.90	6.84	60.38	29,585.69	2n=14C
1981	R2	4,385.00	±	293.35	6.69	11.41	5,593.34	2n=2C
	R3	8,029.00	±	643.78	8.02	20.90	10,241.93	2n=3C
	R4	14,845.00	±	1,553.47	10.46	38.64	18,935.79	2n=8C
	R5	25,730.00	±	4,314.25	16.77	66.98	32,818.32	2n=12C
3903	R2	4,385.00	±	1,239.51	28.26	11.42	5,593.45	2n=2C
	R3	8,556.00	±	1,904.09	22.25	22.27	10,913.23	2n=4C
	R4	17,308.00	±	3,174.93	18.34	45.05	22,076.10	2n=8C
	R5	32,061.00	±	5,313.68	16.57	83.46	40,894.48	2n=15C
3904	R2	3,608.00	±	134.34	3.72	9.39	4,602.15	2n=2C
	R3	6,558.00	±	204.23	3.11	17.07	8,365.36	2n=4C
	R4	12,317.00	±	520.23	4.22	32.06	15,711.25	2n=6C
	R5	22,629.00	±	731.21	3.23	58.90	28,863.29	2n=14C
3908	R2	5,284.00	±	315.86	5.98	13.75	6,739.91	2n=2C
	R3	9,994.00	±	630.90	6.31	26.02	12,747.87	2n=4C
	R4	18,501.00	±	1,089.68	5.89	48.16	23,597.96	2n=6C
	R5	33,888.00	±	405.96	1.20	88.21	43,224.75	2n=13C
PAB	R2	2,054.00	±	107.27	5.22	5.35	2,620.87	2n=2C
	R3	3,756.00	±	133.62	3.56	9.78	4,791.01	2n=4C
	R4	7,349.00	±	101.72	1.38	19.13	9,374.37	2n=8C
	R5	14,008.00	±	280.78	2.00	36.46	17,867.63	2n=14C
PAM	R2	5,978.00	±	874.63	14.63	15.56	7,625.40	2n=2C
	R3	10,888.00	±	1,425.17	13.09	28.34	13,888.38	2n=3C
	R4	20,640.00	±	2,187.50	10.60	53.73	26,326.89	2n=7C
	R5	35,483.00	±	3545.90	9.99	92.37	45,259.17	2n=12C
PSC	R2	1,792.00	±	25.13	1.40	4.67	2,286.72	2n=2C
	R3	3,183.00	±	60.12	1.89	8.29	4,060.04	2n=4C
	R4	5,979.00	±	68.28	1.14	15.57	7,627.06	2n=7C
	R5	11,524.00	±	256.80	2.23	30.00	14,699.72	2n=14C

Phalaenopsis hybrid cultivars: 1702, 1819, 1981, 3903, 3904, 3908; PAB: *P. amabilis*; PAM: *P. amboinensis*; PSC: *P. schilleriana*; SD: Standard Deviation; CV: Coefficient of Variance.

Table 5. The relationships based on Pearson correlation between DNA content and quantitative characteristics.

	DR2R	DR3R	DR4R	DR5R	DR2L	DR3L	DR4L	DR5L	PH	SD	LL	LW	LI	NF	FFL	FFW	SL	SW	PL	PW	ALL	ALW	PL
DR3R	0.99**																						
DR4R	0.95**	0.98**																					
DR5R	0.92*	0.96**	0.99**																				
DR2L	0.91*	0.91*	0.88*	0.88*																			
DR3L	0.93*	0.91*	0.88*	0.87*	0.98**																		
DR4L	0.96**	0.94*	0.90*	0.88*	0.97**	1.00**																	
DR5L	0.97**	0.97**	0.94*	0.93*	0.97**	0.98**	0.99**																
PH	-0.74	-0.70	-0.69	-0.70	-0.84*	-0.90*	-0.86*	-0.82															
SD	-0.83*	-0.80	-0.77	-0.75	-0.93*	-0.96**	-0.94*	-0.90*	0.98**														
LL	-0.68	-0.62	-0.63	-0.65	-0.74	-0.82	-0.77	-0.73	0.98**	0.92*													
LW	-0.43	-0.36	-0.29	-0.33	-0.62	-0.70	-0.65	-0.57	0.82	0.80	0.79												
LI	-0.70	-0.64	-0.61	-0.60	-0.88	-0.89*	-0.82	-0.77	0.99**	0.96**	0.97**	0.85*											
NF	0.42	0.48	0.56	0.62	0.58	0.60	0.55	0.54	-0.78	-0.69	-0.82	-0.54	-0.70										
FFL	-0.65	-0.65	-0.69	-0.72	-0.83*	-0.84*	-0.79	-0.77	0.94*	0.91*	0.91*	0.74	0.88*	-0.92*									
FFW	-0.68	-0.66	-0.69	-0.71	-0.84*	-0.86*	-0.81	-0.78	0.98**	0.93*	0.93*	0.78	0.91*	-0.89*	1.00**								
SL	-0.64	-0.64	-0.67	-0.70	-0.83*	-0.84*	-0.79	-0.76	0.95**	0.92*	0.93	0.78	0.91*	-0.90*	1.00**	1.00**							
SW	-0.65	-0.61	-0.61	-0.63	-0.78	-0.84*	-0.79	-0.74	0.99**	0.94**	0.99**	0.85*	0.98**	-0.83*	0.93**	0.97**	0.96**						
PL	-0.58	-0.60	-0.64	-0.68	-0.80	-0.81	-0.76	-0.73	0.91*	0.88*	0.88*	0.77	0.86*	-0.92*	0.99**	0.99**	0.99**	0.93*					
PW	-0.73	-0.70	-0.71	-0.71	-0.85*	-0.88*	-0.84*	-0.80	0.99**	0.97**	0.97**	0.75	0.97**	-0.82	0.96**	0.97**	0.96**	0.98**	0.92*				
ALL	-0.64	-0.67	-0.74	-0.78	-0.77	-0.78	-0.75	-0.74	0.80*	0.84*	0.90*	0.62	0.82*	-0.96**	0.97**	0.98**	0.96**	0.91*	0.96**	0.92*			
ALW	-0.78	-0.78	-0.80	-0.81	-0.90*	-0.91*	-0.88*	-0.86*	0.97**	0.96**	0.93*	0.70	0.92*	-0.86*	0.98**	0.98**	0.98**	0.95**	0.95**	0.98**	0.96**		
PL	-0.74	-0.74	-0.78	-0.79	-0.88*	-0.89*	-0.83*	-0.83*	0.95**	0.94*	0.92*	0.78	0.90*	-0.89*	0.99**	0.99**	0.99**	0.94**	0.97**	0.97**	0.97**	1.00**	
PW	-0.54	-0.29	-0.30	-0.30	-0.57	-0.60	-0.53	-0.46	0.87*	0.79	0.88*	0.81	0.89*	-0.76	0.85*	0.87*	0.87*	0.91*	0.84*	0.88*	0.77	0.90	0.91

DR2R: DNA content in the R2 peak of root; DR3R: DNA content in the R3 peak of root; DR4R: DNA content in the R4 peak of root; DR5R: DNA content in the R5 peak of root; DR2L: DNA content in the R2 peak of leaf; DR3L: DNA content in the R3 peak of leaf; DR4L: DNA content in the R4 peak of leaf; DR5L: DNA content in the R5 peak of leaf; PH: Plant Height (cm); SD: Stem Diameter (cm); LL: Leaf Length (cm); LW: Leaf Width (cm); LI: Length of Inflorescence (cm); NF: Number of Flower; FFL: Front Flower Length (cm); FFW: Front Flower Width (cm); SL: Sepal Length (cm); SW: Sepal Width (cm); PL: Petal Length (cm); PW: Petal Width (cm); ALP: Apical Lip Length (cm); ALW: Apical Lip Width (cm); PL: Pollen Length (mm); PW: Pollen Width (mm),** significant at $P < 001$; * significant at $P < 005$.

challenging to determine the ploidy level of each cultivar, as most genotypes exhibited cells in diploid, tetraploid, or octoploid conditions.

Correlation analysis between morphological characters and DNA content is available in Table 5. The results show a positive correlation between the DNA contents from different cell ploidy levels, with the higher DNA contents associated with higher ploidy levels. However, DNA contents displayed a negative correlation with morphological characteristics, except for the number of flowers per inflorescence, which exhibited a positive correlation, though not significant. Generally, morphological traits showed a significant positive correlation with each other, except for the number of flowers, which showed a significant negative correlation.

DISCUSSION

Morphological characteristics, such as plant size, plant vigor, flower number, and morphology, are crucial in *Phalaenopsis* orchids breeding to get the progenies with novel features. Utilizing complex hybrids as parental plants in crossbreeding can offer a shortcut to developing novel varieties. However, the success of crosses between complex hybrid varieties has no guarantee due to incompatibility issues between parental plants, leading to post-fertilization embryo abortion (Luo *et al*., 2012). The incompatibility can also arise from differences in ploidy levels or genome size between the parents (Chen and Tang, 2017). Therefore, determining the hybrid parents before crossing is vital to establishing an efficient breeding program. This research

identified the morphological characteristics of six *Phalaenopsis* hybrid cultivars with significant market potential. Three cultivars have the standard large flowers, while the rest are novelty multiflora cultivars. Research results showed significant differences in most morphological characters between these cultivar groups. Crossing these two groups may yield diverse progenies based on these morphological attributes.

On the other hand, using flow cytometry helped analyze the DNA contents of each cultivar. Flow cytometry is a fast and helpful method for determining the ploidy level and an organism's genome size based on the DNA content of its nuclei, particularly in orchids (Hartati *et al*., 2022). It is widely used due to its high throughput, resolution, accuracy, and low operating cost per sample (Garavello *et al*., 2019). Additionally, DNA amounts in nuclei have typical positive correlations with ploidy levels in most plant species (Lee *et al*., 2020). Jones (1996) found that immature leaf tissue provided the most reliable results for several species in *Dendrobium* and *Cattleya,* with endopolyploidy common in leaf cells. Flow cytometry results often exhibit multiple peaks, especially in endopolyploid species (Kron, 2015). High endoreduplication levels have resulted in *Phalaenopsis* spp. and *Doritis pulcherrima* (Chen *et al*., 2011). Endopolyploidy was evident in the leaf and root tissues of *P. aphrodite*. Endopolyploidy occurs in the leaves of diploid and tetraploid seedlings. Four peaks appeared in the histograms of leaves from the diploids, whereas only three were for the tetraploids. The frequencies of nuclei at peaks 3 and 4 and cycle values of the mature leaves were higher than the young leaves (Chen *et al*., 2011).

Teixeira da Silva *et al*. (2014) identified endopolyploid conditions in many flower parts of *Phalaenopsis,* including the dorsal sepal, petal, lateral sepal, labellum, pedicel, column, anther cap, stigma surface, and youngest leaf*.* This experiment analyzed the young leaf and root tip cells. The results suggest a mixoploid condition in the young leaf and root tip cells, with a maximum of 16C. The histogram results of leaf samples showed that the highest nuclear distribution was at peaks R3 and R4 or ploidy patterns 4C and 8C. This research aligns with Lee *et al.* (2007), who observed *P. equestris* leaves, and Jones (1996), who observed *Dendrobium* leaves had more core distribution in 4C and 8C. Root cells showed the highest nuclear distribution, which was stable at the R3 level (ploidy pattern 4C).

Utilizing the ovary part of the flower at the anthesis stage may provide a more accurate determination of cultivar ploidy level to mitigate the high level of mixoploidy in future experiments. Chen *et al*. (2011) reported that the ovary at the anthesis stage produced more high-quality 2C nuclei for measurement by flow cytometry. It is suitable for ploidy determination by flow cytometry because a mature tissue often contains more endopolyploid cells.

Previous research has reported that chromosome and genome sizes among *Phalaenopsis* species vary from 1C = 1.39–8.74 pg (Chen *et al*., 2013; Chen *et al*., 2014; Lee *et al*., 2017). Our flow cytometry results exhibit the genome size of the hybrid cultivar in mean 2C cells from young leaves. For hybrid cultivars 1702, 1819, 1981, 3903, 3904, 3908, and the species PAB, PAM, and PSC, the genome sizes are 5.66, 8.95, 10.26, 11.76, 10.89, 15.42, 4.74, 3.69, and 3.94 pg, respectively. On the other hand, for 2C cells for root tips, the genome size of the above cultivars or species are 8.10, 8.81, 11.41, 11.42, 9.39, 13.75, 5.35, 15.56, and 4.67 pg, respectively. The genome size of hybrid cultivars found in this experiment appears higher than that reported by Lee *et al*. (2020). They found the 2C genome size of standard hybrid cultivars was around 4–6 pg, while novelty multiflora hybrid cultivars ranged from 4 to 14 pg. The genome size of the species PAB, PAM, and PSC was also higher than in the previous report by Chen and Tang (2017). The differences in the results could be due to the different species observed and experimental conditions. Some data showed high coefficient variations (CV > 8%), indicating errors in the DNA extraction or flow cytometry measurement. For example, the leaf

cell samples for species *P. amabilis* (PAB) and *P. schilleriana* (PSC)*,* root cell samples for *P. amboinensis* (PAM)*,* and hybrid cultivar 1819 needed further clarification.

Endopolyploidy, mainly caused by endoreduplication, has reports released on several orchid genera (Vilcherrez-Atoche *et al*., 2022). Endoreduplication refers to the endocycle of the mitotic cell cycle, in which subsequent chromosome separation and cytokinesis do not immediately follow DNA replication (Leitch and Dodsworth*,* 2017; Kobayashi, 2019). This phenomenon complicates the determination of the ploidy level of cultivars or species. Several factors can trigger endopolyploidy, such as tissue type, developmental stage, and genetic factors specific to the species or varieties (Chen *et al*., 2011; Teixeira-da-Silva *et al*., 2014). Additionally, abiotic environmental factors influence endoreduplication, such as light and nutrients (Moluszynska *et al.,* 2013). Temperature can also affect the occurrence of endopolyploidy, such as in the cells of *P. aphrodite* and *Oncidium varicosum* orchids (Lee *et al*., 2007).

Based on chromosome analysis, Lee *et al*. (2020) found that most *Phalaenopsis* cultivars were polyploid, with a few showing hyper- and hypoploid chromosome numbers. Therefore, cytogenetic analysis may still be necessary to elucidate the chromosome constitution, enabling the fixation of the plant ploidy level and proper application of the cultivar stock in plant breeding.

CONCLUSIONS

Morphological characterization highlights significant differences in quantitative traits between standard hybrid and novelty multiflora cultivars. Flow cytometry analysis revealed the mixoploid condition of young leaf and root tip cell nuclei; however, the ploidy level of the hybrid cultivar may be triploid or tetraploid. The correlation between genome size and quantitative morphological characters remains unclear, except for flower numbers that may have a weak positive correlation with the genome size. Further experiments on genome size analysis using other parts of the flower, such as ovary or pollen, and chromosome counting are necessary to determine the accurate plant ploidy level.

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REFERENCES

Chase MW, Cameron KM, Freudenstein JV, Pridgeon AM, Salazar G, Van den Berg C, Schuiteman A (2015). An update on classification of Orchidaceae. *Bot. J. Linn. Soc*. 177: 151–174.

Chen WH, Kao YL, Tang CY (2014). Variation of the genome size among *Phalaenopsis* species using DAPI. *J. Taiwan Soc. Hort. Sci*. 60(2): 115–123.

Chen WH, Kao YL, Tang CY, Tsai CC, Lin TY (2013). Estimating nuclear DNA content within 50 species of the genus *Phalaenopsis blume* (Orchidaceae). *Elsevier J.* 161: 70–75. https://doi.org/10.1016/j.scienta.2013.06.045.

Chen WH, Tang CY (2017). Genome size variation in species of the genus *Phalaenopsis blume* (Orchidaceae) and its application in variety improvement. In: W.H. Chen and H.W. Chen (eds.), Orchid Biotechnology III. World Scientific, Singapore.

Chen WH, Tang CY, Lin TY, Weng YC, Kao YL (2011). Changes in the endopolyploidy pattern of different tissues in diploid and tetraploid *Phalaenopsis aphrodite subsp. formosana* (Orchidaceae). *Plant Sci*. 181: 31–38.http://doi.org/10.1016/j.plantsci.2011.03.006.

Cribb PJ, Schuiteman A (2012). *Phalaenopsis*: Distribution and Ecology. In: Swiss Orchid Foundation (Eds.) Renziana Vol 2: *Phalaenopsis*. 1st edition, Basel: SchweizerischeOrchideenstiftung, pp. 11–13.

De LC, and Pathak P (2015). Value addition in orchids. *J. Orchid India*. 20: 31–37.

Dolezel J, Greilhuber J, Suda J (2007). Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols*. 2(9): 2233–2244. https://doi.org/10.1038/nprot.2007.310.

Escobedo-Garcia-Medrano RM, Burgos-Tan MJ, Ku-Cauich JR, Quiroz-Moreno A (2018). Using flow cytometry analysis in plant tissue culture derived plants. *Methods Mol. Biol.* 1815:317–-332. doi:10.1007/978-1-4939-8594-4_22.

Garavello M, Cuenca J, Dreissig S, Fuchs J, Houben A, Aleza P (2019). Assessing ploidy level analysis and single pollen genotyping of diploid and euploid Citrus genotypes by fluorescence activated cell sorting and whole genome amplification. *Front. Plant Sci*. 10: 1174. https://doi.org/10.3389/fpls.2019.0117.

Hartati S, Sukaya, Samanhudi (2022). Karyotype and ploidy of *Vanda dearie* and *Vanda celebica* orchid using flow cytometry analysis. *J. Agron*. *Indonesia* 50 (3): 337–342. https://dx.doi.org/10.24831/jai.v50i3.43825.

Hinsley A, De Boer HJ, Fay MF, Gale SW, Gardiner LM, Gunasekara RS, Kumar P, Masters S, Metusala D, Robert DL, Veldman S, Wong S, Phelps J (2018). A review of the trade in orchids and its implications for conservation. *Bot. J. Linn. Soc.* 186:435–455.

Jones WE (1996). Determination of nuclear DNA content in orchids by flow cytometry. Thesis for the degree of Master of Science. University of Hawaii, Manoa.

Kobayashi H (2019). Variations of endoreduplication and its potential contribution to endosperm development in rice (*Oryza sativa* L.). *Plant Protection. Sci*. 22(2): 227–241. https://doi.org/10.1080/1343943X.2019.1570281.

Kron P (2015). Endopolyploidy, genome size, and flow cytometry. *J. Int. Soc. Adv. Cytometry* 87(10): 887–889. http://doi.org/10.1002/cyto.a.22718.

Lee HC, Chen YJ, Markhart AH, Lin TY (2007). Temperature effects on systemic endoreduplication in orchid during floral development. *Plant Sci*. 172(3): 588–595. https://doi.org/10.1016/j.plantsci.2006.11.013.

Lee YI, Chung MC, Kuo HC, Wang CN, Lee YC, Lin CY, Jiang H, Yeh CH (2017). The evolution of genome size and distinct distribution patterns of rDNA in *Phalaenopsis* (Orchidaceae). *Bot. J. Linn. Soc*. 185(1): 65–80. https://doi.org/10.1093/ botlinnean/box049.

Lee YI, Tseng YF, Lee YC, Chung MC (2020). Chromosome constitution and nuclear DNA content of *Phalaenopsis* hybrids. *Scientia Horticulturae.* 262: 1–9. https://doi.org/10.1016/j.scienta.2019.109089.

Leitch IJ, Dodsworth S (2017). Endopolyploidy in plants. In: Encyclopedia of Life Sciences. John Wiley & Sons, Ltd, Chichester. https://doi.org/10.1002/9780470015902.a0020097.pub2.

Li C, Dong N, Zhao Y, Wu S, Liu Z, Zhai J (2021). A Review for the breeding of orchids: Current achievements and prospects. *Hortic. Plant J.* 7(5): 380–392. https://doi.org/10.1016/j.hpj.2021.02.006.

Luo YH, Huang ML, Wu JS (2012). Progress in *Oncidium* breeding study. *Acta Agric. Jiangxi* 24: 15–20.

Royal Horticultural Society (2018). Royal Horticultural Society Orchid Hybrid Registration Database Information System. http://apps.rhs.org.uk/horticulturaldatabase/orchidregister/orchidresults.asp/https://doi.org/10.1007/978-3-030-66826-6_10. Accessed: June 2022.

Sattler MC, Carvalho CR, Clarindo WR (2016). The polyploidy and its key role in plant breeding. *Planta* 243(2): 281–296. https://doi.org/10.1007/s00425-015-2450-x.

Sukma D, Aziz SA, Sudarsono S, Romeida A (2015). Pollination success among standard hybrids and Indonesian species of *Phalaenopsis*. *ActaHort*. 1078: 139–148. https://doi.org/10.17660/ActaHortic.2015.1078.19.

Sukma D, Elina J, Raynalta E, Aisyah SI, Aziz SA, Sudarsono, Chan MT (2021). Analysis of the genetic diversity of Phalaenopsis orchids with single nucleotide polymorphisms and snap markers derived from the Pto gene. *SABRAO J. Breed. Genet.* 53(4): 620-631. https://doi.org/10.54910/sabrao2021.53.4.6.

Teixeira da Silva JA, Giang DTT, Dobranszki J, Zheng S, Tanaka M (2014). Ploidy analysis of *Cymbidium, Phalaenopsis*, *Dendrobium* and *Phaphiopedilum* (*Orchidaceae*), and *Spathiphyllum* and *Syngonium* (*Araceae*). *Biologia*. 69: 750–755.

Tomaszewska P, Pellny TK, Hernández LM, Mitchell RAC, Castiblanco V, de Vega JJ, Schwarzacher T, Heslop-Harrison PJS (2021). Flow cytometry-based determination of ploidy from dried leaf specimens in genomically complex collections of the tropical forage grass *Urochloa* s.1. *Genes J*. 12(7): 1–17. https://doi.org/10.3390/genes12070957.

Tsai WC, Dievart A, Hsu CC, Hsiao YY, Chiou SY, Huang H, Chen HH (2017). Post genomics era for orchid research. *Bot Stud.* 58 (61): 2–22. https://doi.org/10.1186/s40529-017-0213-7.

United States Department of Agriculture (USDA) (2018). Floriculture Crops 2017 Summary. United States Department of Agriculture, Washington DC.

Vilcherrez-Atoche JA, Liyama CM, Cardoso JC (2022). Polyploidization in orchids: From cellular changes to breeding applications. *Plants* 11(4): 469. http://doi.org/10.3390/plants11040469.

Willis KJ (2017). State of the World's Plants 2017. Royal Botanic Gardens Kew, London.