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Estimation of DNA content and genome size of some orchids using flow cytometry

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ABSTRACT

Flow cytometry technique is used extensively in plant sciences to reveal the deviation of genomic size to better identify or resolve the closely related organisms or groups. In this study, extracted nuclei from the leaf tissue of ten *Dendrobium* species were analyzed for their DNA content. A flow cytometer was used to measure DNA content after extracting and specific staining of nuclei. Direct comparison was performed using chicken erythrocyte nuclei (1C DNA = 1.25 pg) as a standard. The calculated amount of DNA content showed the variation in the amount, ranging from 3.78 to 8.75pg, equivalent to a genomic size of 1852.20 to 4287.50 Mbp, respectively. There was no correlation between elevation and quantity of genome of studied *Dendrobium* species. It was observed that the quantity of a particular species DNA was inversely proportional to the size of their flowers and positively correlated with the plant height and leaf length. An ANOVA test among the four variables was performed and to identify the subsets that were distinct from one another, we performed Tukey's HSD test. A pair-wise comparison was performed and among the six compressions, only the stem length significantly differed (mean = 97.873, p< 0.001) from other three variables.

Key words: *Dendrobium* species, Eastern Himalayas, floral morphology, genomic DNA.

INTRODUCTION

Dendrobium, with over 1,500 species present in the eastern and southeastern countries of the world is the second-largest genus of the orchid family (Mondal and De, 10). This genus is broad, biologically diversified, and taxonomically complicated. Lokho (8) divided the genus into many sections and sub-sections. There are 124 different *Dendrobium* species in India, making it the country's second-biggest orchid genus (Mondal and De, 10). The widespread distribution, enormous diversity, and great commercial and horticultural worth of *Dendrobium* sp. and hybrids all contribute to the plants widespread fascination (Zhang *et al.*, 19). On the other hand, modern research shows polysaccharides, alkaloids, amino acids, trace elements, and other active ingredients found in *Dendrobium* are pharmaceutically important. Due to the huge diversity at different levels of expression, nowadays *Dendrobium* is considered a model for the study of biodiversity *in-situ* (Mondal and De, 10).

Understanding the nuclear genome size has become crucial in numerous subfields of plant biology, especially taxonomy, systematics, population biology, and ecology. The scientific investigation and understanding of DNA amount, and its impact on organisms has expanded greatly in the past twenty years because of the availability of flow cytometry (Nix *et al*., 12). The 2C nuclear genome can be determined in a fast and easy way using flow cytometry (Dolezel, 2). Thus, the current investigation was designed to examine the amount of DNA present, possible reasons for relative variation of genome sizes, and their correlation with their habitat elevation, flower size, plant height, and leaf length of the selected *Dendrobium* orchids of Eastern Himalayas from their natural habitat of which some are threatened and endangered.

MATERIALS AND METHODS

Ten (10) different epiphytic species of *Dendrobium* (Fig. 1) were collected during their new growth, *i.e.* sprouting of new and fresh leaf time from the different elevations of Darjeeling, Sikkim hills in the season 2021-2022. The details of the collected 10 *Dendrobium* orchid plant materials are listed in Table 1. Roughly, 200 mg of fresh leaf tissue from each *Dendrobium* sp. was procured for the study in flow cytometry. Using the same steps as Galbraith *et al.* (5) and applying Galbraith's buffer, nuclear suspensions were made by slicing leaves and placing them in a petri-plate set on ice. After that, to eliminate the pieces of cells and big debris, the leaf suspension was filtered with a 50µm nylon mesh, and collect 1.5 ml of nuclear solution. Afterward, propidium iodide (50 µg-mL^{-1}) was used to stain the nuclei that had been filtered

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Table 1. Distribution of habitat and floral morphology of *Dendrobium* species.

Species	Sampling location	Distributional range (meter)	Stem length (cm)	Length leaf (cm)	Size of flowers (cm)	Status/ Category
D. longicornu Lindl. Section: Formosae	Lloyd's Botanical Garden, Darjeeling	1300-2500m	60.72 ± 0.75	10.17 ± 0.37	4.47 ± 0.34	Not so uncommon Lucksom (9)
D. densiflorum Lindl. ex Wall. Section: Densiflorum	Lloyd's Botanical Garden, Darjeeling	Maximum 1400m	46.5 ± 1.61	15.98 ± 0.29	5.23 ± 0.21	Common Lucksom (9); Lokho (8)
D. chrysanthum Wallich ex Lindley Section: Dendrobium	Lloyd's Botanical Garden, Darjeeling	1000-2000m	212.76 ± 2.44	17.92 ± 0.39	5.48 ± 0.41	Common Lucksom (9); Lokho (8)
D. nobile Lindl. Section: Dendrobium	Lloyd's Botanical Garden, Darjeeling	900-1800m	88.21 ± 2.08	11.23 ± 0.91	10.94 ± 0.50	Endangered Threatened Lucksom (9); Lokho (8)
D. nobile var. *pendulum* Section: Dendrobium	Lloyd's Botanical Garden, Darjeeling	1500-2000 m	61.13 ± 1.51	9.89 ± 0.33	7.60 ± 0.52	Threatened Lucksom (9)
D. nobile var. *varginalis* Section: Dendrobium	National Research Centre for Orchids, Sikkim	1500 - 2000 m	54.16 ± 0.9	10.05 ± 0.63	7.83 ± 0.30	Threatened Lucksom (9)
D. gibsonii Paxton Section: Holochrysa	National Research Centre for Orchids, Sikkim	750 to 1650m	120.84 ± 1.60	16.0 ± 0.87	5.66 ± 0.40	Rare, Threatened, Endangered Lucksom, (9)
D. moschatum (Banks) Sw. Section: Holochrysa	National Research Centre for Orchids, Sikkim	300 to 900 m	148.46 ± 1.74	4.74 ± 0.51	8.44 ± 0.32	Rare, Threatened, Endangered Lucksom (9)
D. fimbriatum Hook. Section: Holochrysa	National Research Centre for Orchids, Sikkim	1610m and above	179.85 ± 2.12	15.66 ± 0.52	7.83 ± 0.22	Endangered, Threatened, Extremely rare Lucksom (9)
D. rotundatum (Lindl.) Hook.f. Section: Katherinea	Lloyd's Botanical Garden, Darjeeling	1950-2410m	6.10 ± 0.13	7.61 ± 0.52	4.78 ± 0.20	Most common Lucksom (9)

out. To avoid the pigmentation of dsRNA, RNase-A (20 µg-mL^{-1}) was applied to the sample solution. Before analysis, samples were dark-incubated on ice for 60 min. Finally, a flow cytometer (model: iQue® 3, Sartorius*)* examined samples containing propidium iodide-stained nuclei. Chicken erythrocyte nuclei with genome size 2.50 pg were applied as a benchmark for determining nuclear DNA concentration. Every single sample was examined at least three times, with a minimum of 12,000 nuclei analyzed each time. Based on the following calculations, DNA content (pg) (Dolezel and Bartos, 3) and genome size (1 pg = 980 Mbp) (Suda *et al.*, 16) were determined using fluorescence ratios compared to the standard.

$$\text{Sample 2C-DNA concentration (pg)} = \frac{\text{G1-peak mean of sample} \times \text{standard 2C DNA amount}}{\text{G1-peak mean of standard}}$$

C-value (Mbp) = [(2C DNA amount (pg) of sample multiplied by 980 Mbp) ÷ 2]

Statistical analysis was done to find out in-depth results from the calculated data, we performed ANOVA and Tukey's test using the software Statistix version 10.0 (Analytical Software, Florida, USA).

RESULTS AND DISCUSSION

The calculated results of different parameters of ten *Dendrobium* orchids are presented in Tables 1 and 2. The box plot shows the median values of the genome size, length of leaf, stem length, and size of flowers (Fig. 2). The box plot showed that the median value of the stem length was significantly different from other medians. Observations of individual nuclei stained with propidium iodide from young leaves of ten *Dendrobium* species showed two peaks that correspond to the G_0/G_1 phase and G_2 + M (M = mitosis) phase of the cell cycle were also detected (Fig. 3). The concentration of DNA has been displayed by a scatter graph corresponding to

Fig. 1. Close-up view of plants and flowers of ten *Dendrobium* species.

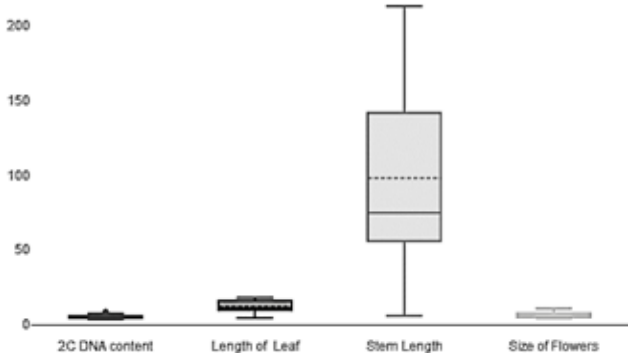

Fig. 2. Box and whisker plot showing data distribution of genome size, length of leaf, stem length, and size of flowers of ten *Dendrobium* species.

SSC-A/FSC-A (Fig. 4). The amount of DNA content ranges from 3.78 ± 0.03 to 8.75 ± 0.20 pg, which is equivalent to a genomic size of 1852.20 to 4287.50 Mbp, respectively (Table 2) of the *Dendrobium* species under investigation. The minimum quantity of DNA content was noticed in *D. densiflorum* (3.78 ± 0.03 pg), whereas the highest one was obtained for *D. gibsonii* (8.75 ± 0.20 pg).

In the present study, the quantity of DNA was inversely proportional to the flower size (r = - 0.138), DNA concentration was positively correlated with plant height (r = 0.497) and the correlation between leaf length and DNA content was poorly positive (r = 0.243) (Table 3). The Shapiro-Wilks (W) test showed that the genome size, leaf length, stem length, and flower size data followed a normal distribution. It was assumed that further parametric tests could be conducted. Significant mean differences (F = 18.65, p

Table 2. 2C DNA content, genome size, and chromosome number of ten *Dendrobium* species.

Species	Median (FL2-A)	2C DNA content (pg)	1C Genome size (Mbp)	No. of chromosomes (2n = 2x =)	DNA amount/ chromosome (pg)
Dendrobium longicornu Lindl.	132667	5.89 ± 0.03	2886.10 ± 14.70	38	0.155
Dendrobium densiflorum Lindl. ex Wall.	85100	3.78 ± 0.03	1852.20 ± 14.70	38,40	0.099, 0.94
Dendrobium chrysanthum Wallich ex Lindley	169000	7.50 ± 0.09	3675.00 ± 44.10	38,40	0.197, 0.187
D. nobile Lindl.	121500	5.40 ± 0.09	2646.00 ± 42.20	38,40	0.142, 0.135
D. nobile Lindl. var. *pendulum*	114500	5.08 ± 0.09	2489.20 ± 44.10	38,40	0.133, 0.127
D. nobile Lindl.var. *varginalis* Hort.	106000	4.71 ± 0	2307.60 ± 40.00	38,40	0.123,0.117
Dendrobium gibsonii Paxton	197000	8.75 ± 0.20	4287.50 ± 98.17	38	0.230
Dendrobium moschatum (Banks) Sw.	140333	6.23 ± 0.07	3052.70 ± 34.30	38,40	0.163, 0.155
Dendrobium fimbriatum Hook.	93167	4.14 ± 0.08	2028.60 ± 39.20	38,40	0.108, 0.103
Dendrobium rotundatum (Lindl.) Hook.f.	97800	4.34 ± 0.07	2126.60 ± 33.24	40	0.108
Chicken erythrocyte (internal standard)	56300	2.50 ± 0.14	1225.00 ± 68.60	-	-

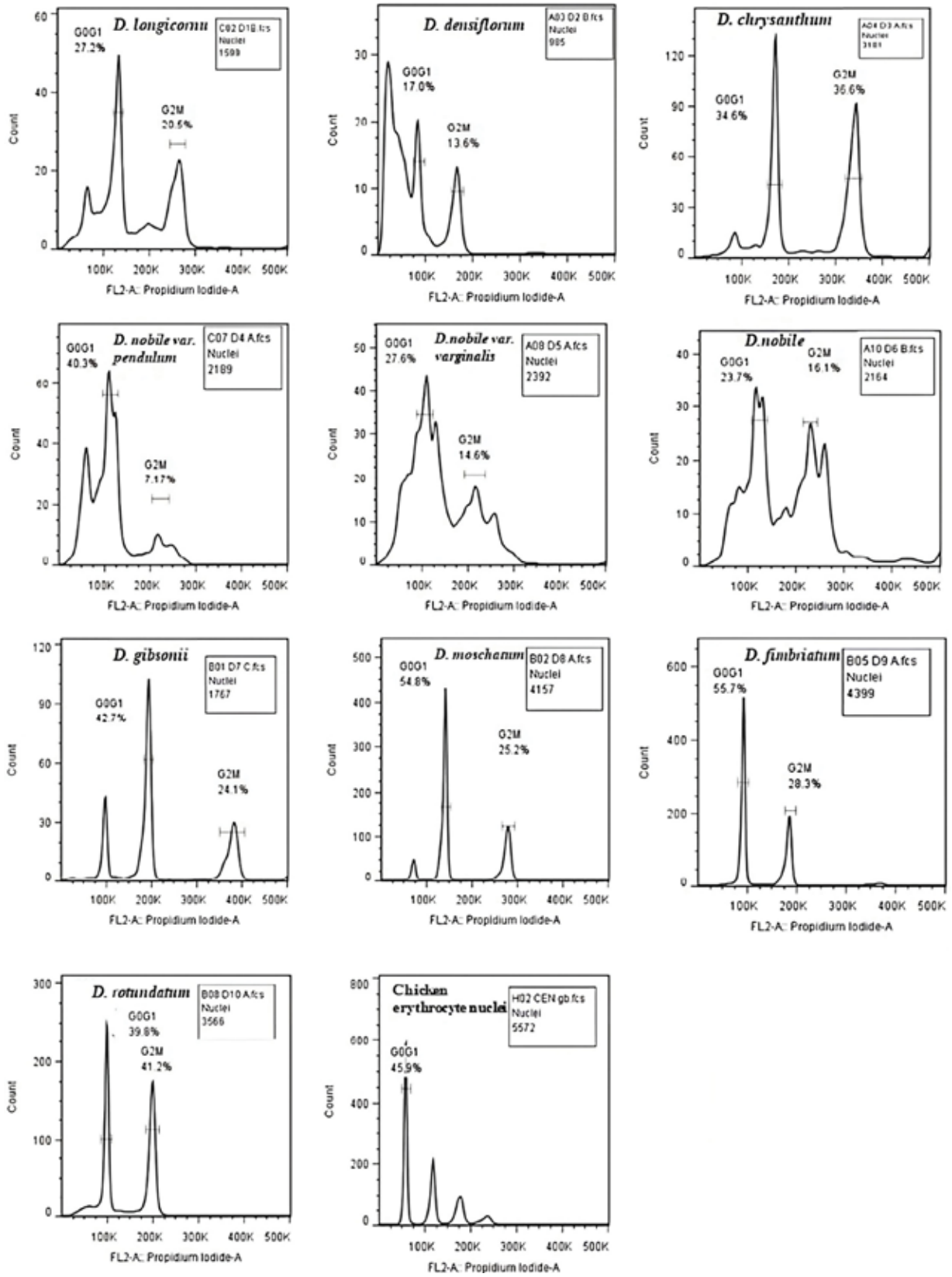

Fig. 3. G_0/G_1 and G_2 + M peaks of ten *Dendrobium* species and the last one is the standard.

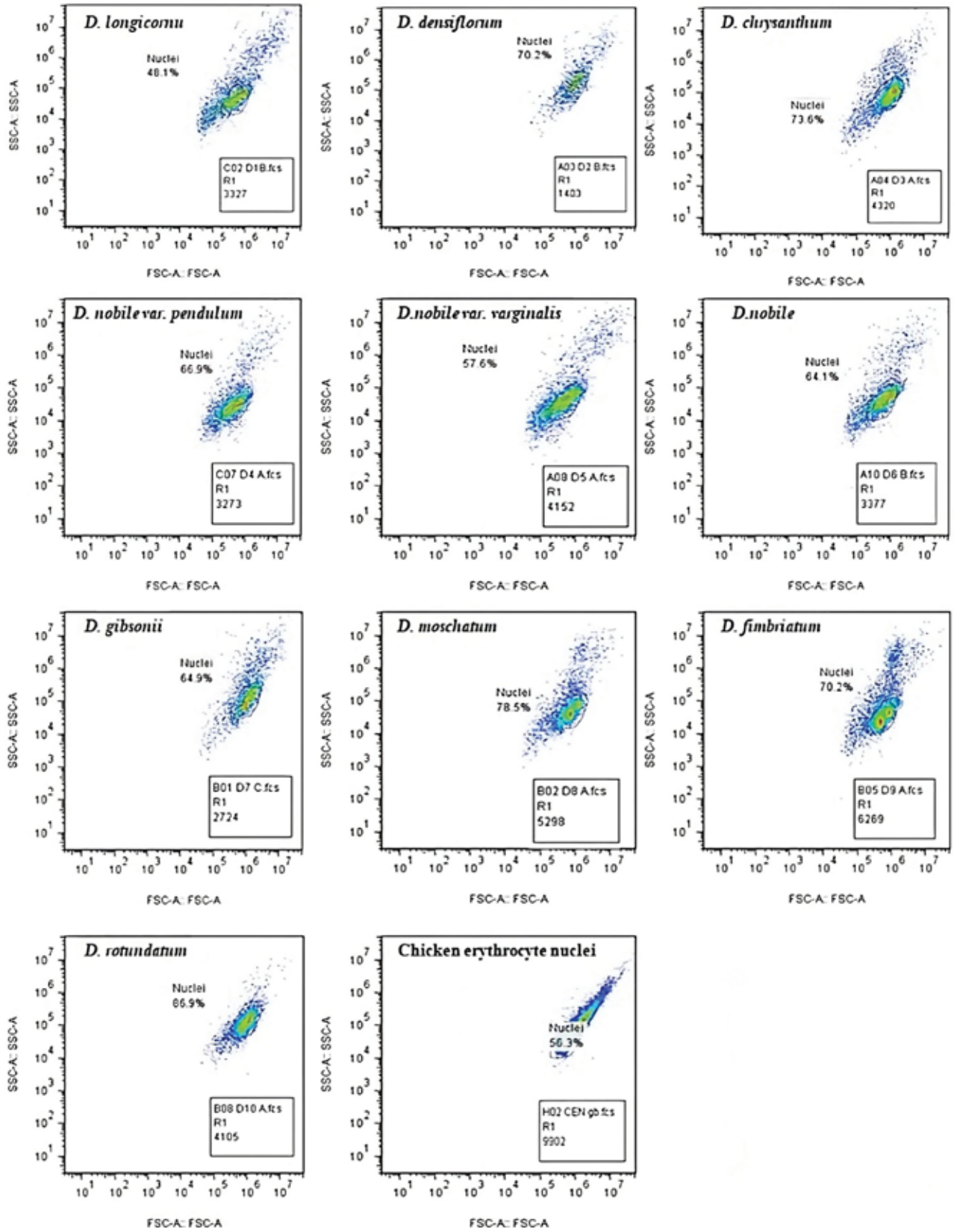

Fig. 4. Scatter plot (SSC-A/FSC-A) of ten *Dendrobium* species and a standard value.

< 0.0001) were found using an ANOVA test between the four variables (Table 4). To identify the subsets that were distinct from one another, we performed Tukey's HSD test. A pair-wise comparison was performed and among the six compressions only the stem length significantly differed (mean = 97.873, p< 0.001) from the other three variables (Table 5).

The current research presents whole-genome flow cytometry data for ten *Dendrobium* species. The genome size variation was found among the selected species of *Dendrobium*, revealing that it ranged from 3.78 to 8.75 pg, and correlated with the generalized value of earlier reports. In an earlier study, among 91 orchid species, the calculated C-value ranges from 0.33 to 55.4 pg with the mode, and average values are 1.2 and 8.5 pg, respectively (Leitch *et al.*, 7). The analysis of 149 orchid species revealed significant dispersion in genome size (1C), ranging from 341 to 54,878 Mb (Trávníček *et al.*, 17). Our findings fall within the aforementioned large range of genome size and modest to average value range. Earlier, Narayan *et al.* (11) documented five *Dendrobium* species that have their DNA content quantified; the amount ranging from 5.1 to 11.7 pg. This result supports our findings closer to these results. The 37 *Dendrobium* species studied have a wide variation in 2C DNA content, from 1.53 to 4.23 pg (Jones *et al.*, 6). As the big genome, size is not explained by ploidy variations; but supports possible ancient origins like *Peristeria elata* (Dressler, 4). Furthermore, 15 Polish orchid species had their nuclear genome amounts tested, and the results ranged from 14.15 to 82.10 pg (Rewers *et al.*, 14). Despite having a fixed chromosomal number of 2n = 18, the amount of diploid DNA in the eight species of Mediterranean *Anacyclus* ranges from 9.37 to 15.69G bp (Vitales *et al.*, 18). In our study, the ten *Dendrobium* species have chromosome numbers either 38 or 40, which is almost similar, but genome size varies significantly among the selected species. The probable reasons for genome size differences among selected *Dendrobium* species may be caused by changes in the quantity of heterochromatin, deletion or duplication of chromosomal parts, and changes in the sequence number of palindromic DNA segments or by transposons, non-coding and repetitive DNA, proliferation, and multiplication of satellite genome repetition, or rearrangement of chromosomes (Vitales *et al.*, 18). Uneven homologous recombination and unlawful recombination also contribute to fascinating

Table 3. Correlation among the four variables of *Dendrobium* species.

Variable	Genome size (pg)	Leaf length (cm)	Stem length (cm)	Flower size (cm)
Genome size	1			
Leaf length	0.243	1		
Stem length	0.497	0.452	1	
Flower size	-0.138	-0.261	0.188	1

Table 4. ANOVA table showing the significant mean difference among the variables.

Source	SS	df	MS	F	F-crit	P value	Eta-sq	RMSSE	Omega sq
Between groups	60655.06	3	20218.35	18.65067	2.8662	< 0.0001	0.608491	1.365674	0.56967
Within groups	39025.99	36	1084.055						
Total	99681.05	39	2555.924						
Grand mean = 30.552							CV = 107.77		

Table 5. Tukey's pairwise Honesty Test showing significant differences among variables and levels of significance.

Variable	Pairwise honestly significant difference (mean difference)			
	Genome size	Leaf length	Stem length	Flower size
Genome size	1	6.34	92.29***	1.24
Leaf length		1	85.95***	5.1
Stem length			1	91.05***
Flower size				1
Critical value for comparison 39.665 (HSD = 0.05)			*** = p < 0.001	

results for C-value reduction in some angiosperms (Bennetzen *et al.*, 1). The genomic size of nuclear DNA and altitude correlate positively, found in *Allium* sp., negatively correlated with maize, and no correlation was found in *Brachypodium* sp. (Oney *et al.*, 13). The current investigation also aimed to find a link between the gradually increasing elevation of habitat and the amount of DNA of *Dendrobium* species. The results of such a study also support the no correlation view because *Dendrobium nobile* var. *varginalis* growing at 1500-2000 m altitude contains the lowest amount of DNA (4.71 pg), whereas *Dendrobium gibsonii* distributed in the 750-1650 m altitude contains a higher amount of DNA, *i.e.* 8.75 pg. Therefore, there was no correlation found between elevation and quantity of genome of studied *Dendrobium* species. Among five sections of *Dendrobium* species, one species under sect. Holochrysa and one species under sect. Dendrobium, contain the highest amount of DNA. However, one species is under sect. Formosae and three species under sect. Dendrobium contains an intermediate amount of DNA. A low amount of DNA was noted in one species in each sect. Densiflorum, Holochrysa, and Katherinea respectively. Larger-genome plant species have less genetic variation, are less diversified, and are more likely to go perish (Rewers *et al.*, 14). Among the ten selected species, six *Dendrobium* species are uncommon, possibly because of the destruction of natural homes, anthropogenic activity, and a shift in climate like some other plants (Smallwood and Trapnell, 15). In this study, we find that the number of genomes is associated either positively or negatively with the size of flowers, plant height, and leaf length. Few quantitative and qualitative morphological, anatomical, biochemical, and horticulturally relevant characters (such as the size of seed, leaf, *etc*.) in orchids are associated with the size of their genome (Rewers *et al.*, 14). According to Tukey's pair-wise observation, changes in genome size and other observed characteristics have a significant effect on average stem height. Earlier finders came to the same point that Genome size strongly influences the plant height, length of leaf in *Helianthus tuberosus*, (Zhan *et al.*, 20), and other morphological traits in *Festuca* sp.

In the present study, the genome size varied among the tested *Dendrobium* species. The variation was also found in the stem length, length of leaf, and size of flower, despite having the same chromosome numbers. The measured quantitative characters were either positively or negatively correlated except between genome size and altitude.

AUTHORS' CONTRIBUTION

Conceptualization (AM, KKD); Designing of the experiments (AM, KKD); Execution of field/lab experiments and data collection (AM); Analysis of data and interpretation (AM, KKD); Preparation of the manuscript (AM, KKD).

DECLARATION

There is no conflict of interest in this manuscript.

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