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Research Article
Diversity in Morphology and Growth Characteristics of *Dendrobium anosmum* Variations in Lam Dong, Vietnam

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Abstract

Background and Objective: *Dendrobium* is a high value for ornamental and decorative plants with different variations in nature. However, identify *D. anosmum* and *D. parishii* species was difficult because of the similar morphology. This study was done to evaluate the growth cycle, compare morphology and use the molecular identification method to determine three different morphological forms of Gia Hac belonging to *Dendrobium anosmum*. **Materials and Methods:** In this study, *Dendrobium* (ninety variations) were in Di Linh district (Lam Dong, Vietnam) including white (TB-12 Dalat), pink (TB-15 Dalat) and purple (TB-16 Dalat) for morphological and molecular identification. Their young shoots were disinfected and used as original material for micropropagation. **Results:** Based on morphology, the purple flower were observed to be similar to *D. anosmum*, meanwhile, the pink flower and white flower were similar to *D. parishii*. However, the analysis and construction of phylogenetic plants via ITS sequences showed that three groups had 100% homogeneous similarity to the ITS region of *D. anosmum* rather than *D. parishii*. Besides, *in vitro* propagation procedures of young shoots of three different morphological forms were designed in this study. **Conclusion:** These results were significantly contributed to the diversity control and micropropagation of *Dendrobium* variations, serving for plant conservation and providing new sources for the orchid market.

Key words: *Dendrobium anosmum*, ITS region, molecular identification, morphological characteristics, regeneration rate

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Orchid family called Orchidaceae is the second largest family of flowering plants kingdom including more than 25,000 species distributed in 880 genera[1]. *Dendrobium* is one of the largest genera of Orchidaceae, consisting of nearly 1600 species[2]. Vietnamese orchids are considered to be very diverse and rich for many beautiful species with medicinal and commercial values. Among them, *Dendrobium anosmum*, in the wild, called Gia Hac orchid is not only gives beautiful flowers but also has a great fragrance. This orchid has droopy stems, large single flowers, pointed flamingo petals and purple-striped lips, whereas *Dendrobium parishii* named "Song Hong" has standing or elongated stems, purple-flamingo flowers, two lips with dark reddish marks, the posterior part with red horizontal stripes was described morphologically[3] and has some variations such as pale pink, dark pink or white petals with purple lips, white flowers[4]. The flowers of these orchids are both gorgeous and have great fragrances. Therefore, they have become the target of many people, so they are on the verge of extinction[5].

Nowadays, it is difficult and confusing for researchers to identify two species both *D. anosmum* and *D. parishii* if they are based on the similar morphological characteristics of the two groups. As a result, this has made many buyers and tissue culture companies that want to massively multiply these precious orchids confused. Currently, the molecular identification method has been used to accurately determine genetic and phylogenetic relationships. Specifically, the groups *D. parishii* and *D. anosmum* are always two monocyclic species (that means they always form a sister cluster)[6]. Furthermore, *D.* aff. *anosmum*, a variant that has similar morphology to *D. anosmum* but is more genetically related to *D. parishii* than the *D. anosmum* group was observed. Before that, the ITS region sequence to identify the *D. parishii* and *D. anosmum*[7]. They have identified several variants with *D. anosmum* morphology but are closely related to *D. parishii*. However, they have not concluded yet, compare to research on *D.* aff. group. *anosmum* as published by researchers[6].

Presently, *in vitro* culture has been used to conserve and propagate some endemic and rare species of orchids that provide high commercial value. Therefore, this study was done to evaluate the growth cycle, compare morphology and use molecular identification method to determine three different morphological forms of Gia Hac belonging to *Dendrobium anosmum* or *Dendrobium parishii* group, meanwhile, using *in vitro* culture method to conserve and propagate these orchids rapidly.

MATERIALS AND METHODS

Explant collection: Ninety samples including three different morphological forms of flower including white (blooming in December-January, 2017), pink (blooming in April-May, 2017) and purple (blooming in February-March, 2017) of Gia Hac orchid were collected from their natural habitat in Di Linh district, Lam Dong province and planted at Tay Nguyen Institute for Scientific Research (Dalat city, Lam Dong province, Vietnam). They were designated as TB-12 Dalat, TB-15 Dalat and TB-16 Dalat (Fig. 1a-c), respectively. Their young shoots with a height of 2-3 cm were disinfected to provide the starting material for micropropagation. The time of growth cycle observation and experimental design was from January, 2018-December, 2020.

Evaluation of the growth cycle, blooming season and structure of flowers of three differently morphological forms: The growth cycle of three differently morphological forms was observed continuously for three consecutive years. Data have been recorded since the orchids started to sprout and finished when they bloomed. The size of different parts of their flowers consisting of dorsal sepal, lateral sepals, petals and lip) (Fig. 1a1-c1), pollen (Fig. 1a2-c2) and column (Fig. 1a3-c3) were measured and compared.

Molecular identification using ITS sequences: For DNA sequence analysis, we based on a completely established procedure according to researchers[7] on orchids. This procedure includes DNA extraction, amplification of ITS region, sequencing and nucleotide analysis of the ITS. Genetic distance was calculated based on this ITS fragment and the phylogenetic tree was constructed using Maximum Likelihood (ML) in MEGA 6.0 software.

Evaluation of the ability to shoot regeneration from young shoots having three different morphological forms: Young shoots with a height of 2-3 cm of three morphological forms were sterilized with 0.1% $HgCl_2$ for 7 min, then rinsed with sterile distilled water 5 times. Next, remove the surrounding leaves and cut off the outer dead cells caused by the disinfectant. Two culture media including MS and SH supplemented with 2.0 mg L^{-1} BA, 9 g L^{-1} agar, 30 g L^{-1} sucrose and 1 g L^{-1} activated charcoal, pH = 5.8 were used. Monitoring parameters included the number of survival explants, the number of dead explants was recorded. All culture media were incubated at 25±3°C temperature under 16 hrs/day photoperiod with 2,500-3,000 lux light intensity and 75-85% humidity.

Fig. 1(a-c): Differences between three different morphological forms of *Dendrobium anosmum*, (a) *Dendrobium* aff. *anosmum*-pink flowers, (b) *Dendrobium* aff. *anosmum*-purple petals and (c) *Dendrobium* aff. *anosmum*-white flowers
a1, b1, c1: Dorsal sepal, lateral sepal, petal, lip, a2, b2, c2: Pollinia and a3, b3, c3: Column

Evaluation of the effects of BA and NAA concentration on the ability to shoot growth and development of three differently morphological forms: *In vitro* shoots of three morphological forms derived from regeneration experiment with a height of about 1-1.5 cm were cultured on SH medium supplemented with 1.0 mg L^{-1} BA, NAA (0, 0, 5, 1.0, 1.5 mg L^{-1}), 9 g L^{-1} agar, 30 g L^{-1} sucrose and 1 g L^{-1} activated charcoal. Monitoring parameters included shoot height (cm), number of leaves per shoot, number of roots/buds and shoot morphology were collected. All culture media were

incubated at 25±3°C temperature under 16 hrs/day photoperiod with 2,500-3,000 lux light intensity and 75-85% humidity.

Data processing: All experiments were arranged completely random with 1 factor, 3 repetitions. Data were processed by statistical analysis software SPSS 16.0 by Duncan test method with $\alpha = 0.05$.

RESULTS AND DISCUSSION

Growth cycle, blooming season and structure of flowers having three different morphological forms: After three consecutive years of studying on the growth and development of three differently morphological forms, the results showed that there were some remarkable differences in the time of new shoot formation, the average of leaf width and leaf length, deciduous season, blooming season, number of flower buds per node and fragrance. In particular, new shoots formed as early as February, every year on white flowering plants (erect stems), followed by purple flowering plants (droopy stems): Their shoots started from February-March and final shoots of pink flower (erect stems) appeared from April each year. The deciduous season on the orchid group (*Dendrobium*) in Vietnam is heavily influenced by the weather, especially the rainy and sunny seasons. Normally, the growth cycle of this orchid group begins at the beginning of the rainy season and ends at the end of the rainy season, then, they shed their leaves and bloom in the spring.

In this experiment, the deciduous season was recorded to be significantly different, specifically, the group of white flowers fell around October, purple was deciduous in November and December and the pink group was deciduous around January, in the following year. The deciduous season was greatly affected by the duration of the rainy season at the end of the year. In other words, the orchids only shed their leaves when the rainy season ends. Besides, the blooming season was also different. Normally, about two months after the deciduous season, they begin to bloom. In this study, specifically, white flowers bloomed in December and in early January of the following year. Purple flowers bloomed in February and March of the following year and pink flowers bloomed the latest in April and May, of the following year (Table 1).

Current research on leaf width and length, flower colour of white-purple flowers *D. anosmum* and pink Song Hong (*D. parishii*) was consistent with the description of Ho PH[3].

Table 1: Growth cycle, blooming season, and structure of flowers of three differently morphological forms (*D. anosmum*)

Stems and flowers	Growth cycle	Average leaf width and length (cm)	Deciduous season (month)	Blooming season (month)	Number of buds/node	Fragrance	Capsule/fruit
TB12-Dalat	Green erect stems, white petals	Shoots started to develop in February	2.53-9.05	October	End of December-January the following year	1-2	Light scent	3-4 cm green, long capsule
TB15-Dalat	Brown-purple erect stems, pink petals	Shoots started to develop in April-May	3.07-10.05	January	April-May	1-3	Deep scent	3-4 cm, brown-purple long capsule
TB16-Dalat	Brown-purple drooping stems, purple-white petals	Shoots started to develop in February-March	2.55-13.50	November	February-March	1-3	Light scent	4-5 cm brown-purple long capsule

Table 2: Compare the size of different parts of flowers of three differently morphological forms

Forms	Stem and flower	Parts of flower									
		Dorsal sepal (cm)		Lateral sepal (cm)		Petal (cm)		Lip (cm)		Column (cm)		Pollinia (mm)
		Length	Width	Length	Width	Length	Width	Length	Width	Length	Width	Length
TB15-Dalat	Brown, purple erect stem, pink petals	2.46^{c}	1.00^{b}	2.52^{b}	1.32^{c}	2.81^{c}	1.13^{c}	2.45^{b}	1.72^{c}	1.45^{b}	0.41^{c}	21.66^{c}
TB16-Dalat	Brown, purple drooping stem, purple-white petals	3.30^{b}	1.21^{a}	3.21^{a}	1.89^{a}	5.45^{a}	1.25^{b}	4.01^{a}	2.34^{a}	2.10^{a}	0.51^{b}	26.66^{b}
TB12-Dalat	Green erect stem, white petals	3.56^{a}	1.32^{a}	3.25^{a}	1.61^{b}	3.50^{b}	1.51^{a}	3.90^{a}	2.25^{b}	2.03^{a}	0.61^{a}	30.66^{a}

*Different letters (a, b, c···) in the same column indicate significantly different means using Duncan's test ($\alpha = 0.05$)

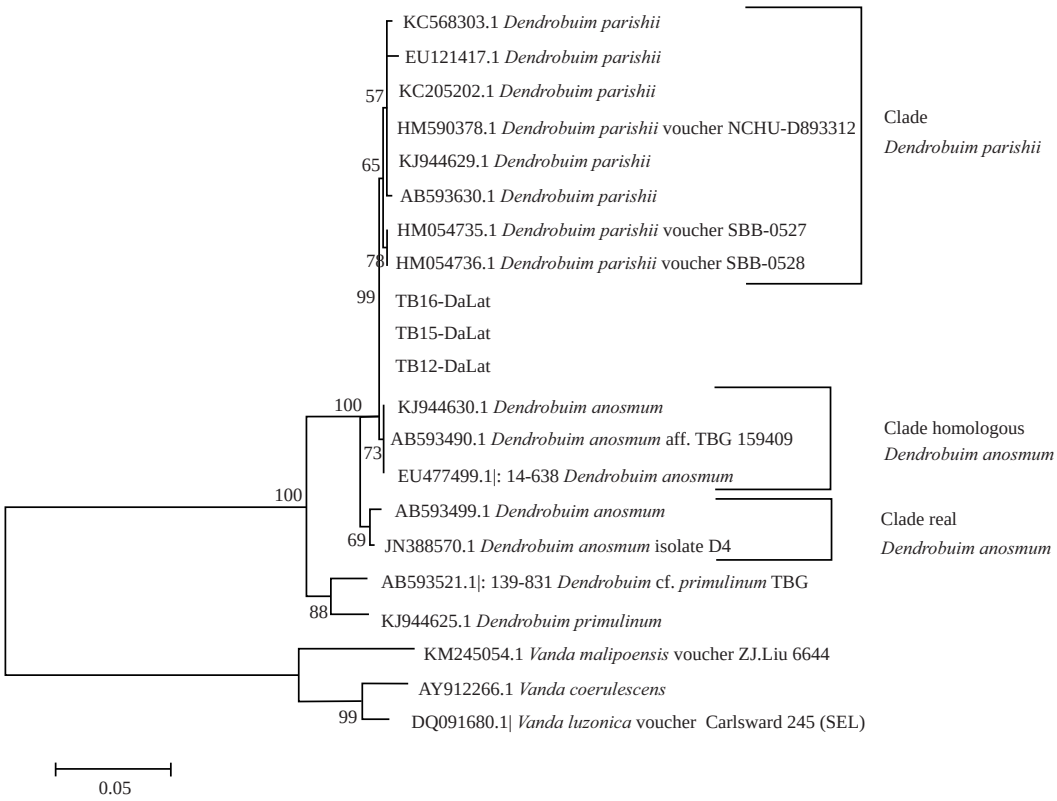

Fig. 2: Maximum likelihood (ML) phylogenetic tree constructed from the dataset of 21 sequences containing 588 bp length
Optimal evolutionary model was T92+G (-ln = 1830.249), bootstrap value at 500 repetitions. The distance rate represented the number of changes at each position

Additionally, purple-white flowers showed some differences in the number of flowers per node. From 1-3 flowers per node were observed, whereas, according to the description of Pham Hoang Ho, this species has single flowers. More interestingly, all three forms had great fragrances which make high value for this group of orchids. The white flowering plants were quite similar in morphology such as erect stems, leaf morphology and the structure of the flowers to the pink flowering plants. However, white plants bloomed larger flowers. That makes them exceedingly rare in nature, leading to fewer notices in any reports on taxonomy. Based on the morphological characteristics obtained, we concluded that white flowering plants may be a variant form of pink flowering plants (*D. parishii*). When it comes to the sizes of the different parts of the flower, there were some remarkably marked differences in the length of dorsal sepal, petals and pollen. In addition, the width of lateral sepals, petals, lips and column of three morphological forms (Table 2, Fig. 1) was also significantly different. Hoa reported that the 3 groups (*D. anosmum, D. aphyllum* and *D. parishii*) are very similar in morphological form and have close evolutionary relationships, so they can be cross-pollination produces hybrids. Therefore, based on morphology, the morphological diversity and vegetative similarity between species for taxonomy were always difficult[8,9].

Molecular identification using ITS sequences: Although the three collected orchid samples had similar morphology to the described species *D. parishii*, their flower colours varied from white to purple or pink and the end of the flower simultaneously appeared erect and droopy which characters have never been observed before from the original *D. parishii*. Furthermore, the white colour of this species is also a feature beyond the recognition of Dendrobium taxonomy documents. Therefore, the ITS regions were sequenced and analyzed to find out the molecular bases for the species identification of these 3 orchid samples. The results showed that the ITS region lengths of 3 sequences TB-12, TB-15 and TB-16 were 695, 723 and 720 bp, respectively, which covering the entire analyzed regions of ITS1, 5.8S and ITS2. All these 3 sequences were 100% identical and they were shown to be either *D. parishii* or *D. anosmum* using the BLAST test (Fig. 2). From the BLAST

results, we selected the sequences with similarity in the range of 97-100% for analysis and 3 more other samples of Vanda species were also chosen as outgroup control. In total, the data set contained 21 sequences including 588 nucleotide positions after eliminating blanks and ambiguity areas. The optimal evolutionary model was T92+G with -ln = 1830.249.

Phylogenetic tree constructed using ML algorithm with 500 repetitions (Fig. 1) presented that *D. parsashii* and *D. anosmum* formed a single clade as described previously[6]. In which, the *D. anosmum* group was divided into two separate clusters: the original real *D. anosmum* cluster (including AB593499 and JN388570) located at the late divergence and separated from the *D. parishii* group, meanwhile the species *D.* aff. *anosmum* with similar morphology to *D. anosmum* (but not *D. anosmum*) was in the same cluster with *D. parishii* (the homologous *D. anosmum*). The location of these three samples that were neither in the cluster with *D. parishii* nor in the adjacent *D. anosmum* cluster in this study was a new finding.

For genetic distance calculation (p), the 3 Dalat samples showed genetic distance at 0.00314 (equivalent to 0.314%) with the *D. parishii* group and 0.0017 (equivalent to 0.17%) with the homologous *D. anosmum* cluster and at 0.0179 (equivalent to 1.79%) with the real *D. anosmum* clusters. This suggested that the Dalat group had a close relationship with the *D.* aff. *anosmum* group (homologous *D. anosmum*) rather than with *D. parishii*. However, because the genetic difference was quite small (0.00314 and 0.00179) it was not sensitive enough to conclude the exact species name for 3 Dalat samples. Hence more markers in the non-coding regions such as trnH-psbA, atpH-atpF need to be analyzed to determine their exact species names. The ITS analysis results of the three Dalat samples also indicated that the two species *D. parishii* and *D. anosmum* have a remarkably similar appearance, even flower colour and stalk characteristics cannot become indicators of distinct identification of species.

Evaluation of the ability to shoot regeneration from young shoots of three differently morphological forms: The young shoots of three differently morphological forms were cultured on SH and MS media after 60 days (Fig. 3a-c), the rate of regenerated explants was significantly different. A minimum survival rate of 40% was observed on shoot explants of dark pink flowers. Mortality was as high as 60%, mostly since the culture was critically contaminated with bacteria. In the nutrient medium, infectious bacteria located deep in the vasculature grew rapidly and formed mucous membranes surrounding the culture and separating explants from the culture. Meanwhile, explants of the purple flower were observed to have a higher regeneration rate (60%) and shoot growth on SH medium (Table 3 and Fig. 3a1-c1), fewer dead explants and fewer internal bacteria. The highest regeneration rate was obtained on white flowering plants (80%) on SH medium, the internal infection on these explants was low (20%). Necrosis explants, in this case, were caused by a culture method that had carried fungi and bacteria from the outside into the culture flask.

Our results for the high rate of shoot regeneration were also consistent with the study[10] that indicated using spring shoots resulted in the highest regeneration rate. To create a starting material source for *in vitro* studies on orchids that originated from nature, most researchers have chosen to use the seeding method and use these seedlings for further studies[11-14].

Effects of BA and NAA concentrations on the ability to regenerate shoots of three differently morphological forms: After 90 days of culture, on all three different morphological forms, the results showed that shoot growth and development *in vitro* still had similar morphological characteristics with those of the parent orchids. At the concentration of 0.5 mg L^{-1} NAA, all monitoring parameters of three differently morphological variations including shoot height (4, 3.7, 4.4 cm), the number of leaves/shoot (5, 4.3, 5 leaves), the number of roots per shoot (7 roots) (Table 4) were recorded. They grew and developed superiorly, compared to the control treatment. When NAA concentration was increased to 1.0 mg L^{-1}, the monitoring parameters did not differ significantly from the 0.5 mg L^{-1} concentration. Meanwhile, at a concentration of 1.5 mg L^{-1} NAA, we observed slow growth and development of some monitoring parameters like shoot height. However, there were no significant differences in the number of leaves/shoot and the number of roots/shoot as shown in Table 4 and Fig. 3(a2-c2).

In particular, there was a growing abnormality in the morphology of the roots. They were short and swollen, which was detrimental to the domestication of the seedlings at the nursery stage. The results of this study showed that parameters such as shoot height, number of roots/shoot were higher than those reported previously[14], who only achieved (0.98 cm/shoot and 5.88 roots/shoot), however, the number of leaves/shoot was lower (7.63 leaves/shoot). This difference

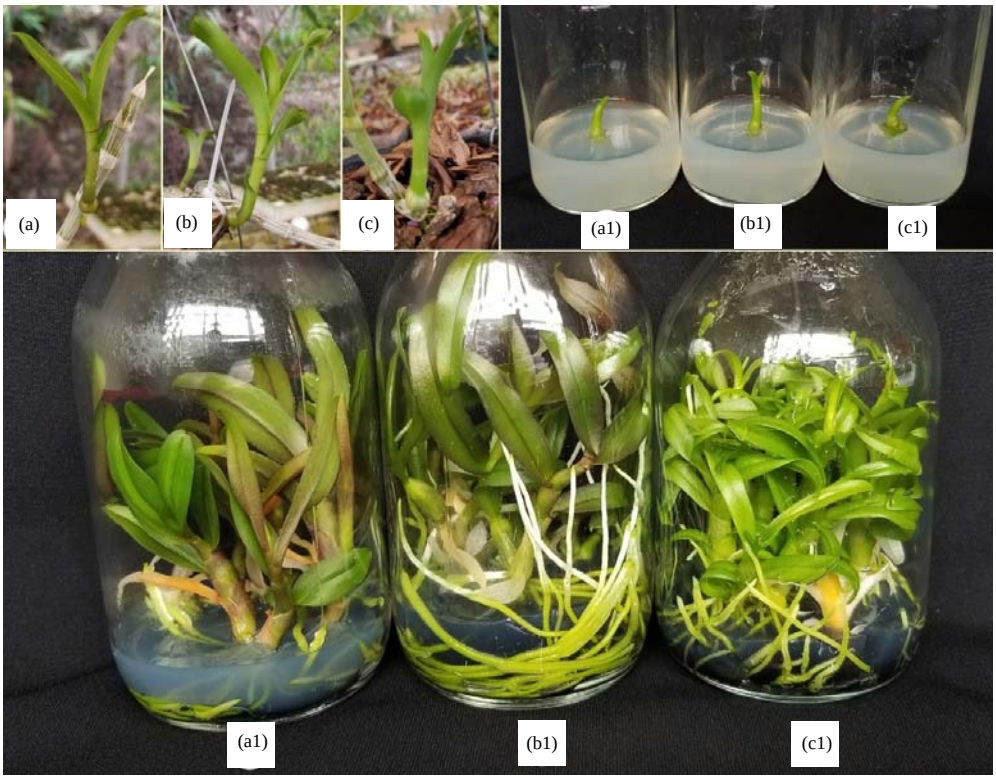

Fig. 3(a-c): Shoot regeneration and complete plantlets of 3 different morphological forms. *Ex vitro* young shoots (a) TB15-Dalat, (b) TB16-Dalat and (c) TB12-Dalat

a1, b1, c1: *In vitro* regenerated shoots of 3 forms (TB15-Dalat, TB16-Dalat, TB12-Dalat) and a2, b2, c2: Plantlets derived from TB15-Dalat, TB16-Dalat TB12-Dalat

Table 3: Compare the ability of shoot regeneration of three differently morphological forms after 60 days of culture

Culture media	Shoots	Number of explants	Survival explants (%)	Necrotic explants (%)
MS	TB12-Dalat	10	70.00^{b}	30.00^{bc}
	TB15-Dalat	10	40.00^{c}	60.00^{a}
	TB16-Dalat	10	53.33^{bc}	46.67^{ab}
SH	TB12-Dalat	10	80.00^{a}	20.00^{c}
	TB15-Dalat	10	40.00^{c}	50.00^{a}
	TB16-Dalat	10	60.00^{b}	40.00^{b}

*Different letters (a, b, c⋯) in the same column indicate significantly different means using Duncan's test ($\alpha = 0.05$). MS: Murashige and skoog medium (1962) and SH: Schenk and Hildebrandt (1972)

Table 4: Effects of BA and NAA concentration on the growth and development of shoots of three differently morphological forms after 90 days of culture

PGRs (mg L^{-1})				Number	Number	
			Shoot	of leaves	of roots	
BA	NAA	Forms	height (cm)	/shoot	/shoot	Shoot morphology
1.0	0	TB12-Dalat	2.54^{e}	3.51^{b}	3.67^{cd}	Green shoots, light green stem
	0.5	TB12-Dalat	4.03^{b}	5.03^{a}	7.01^{a}	Green, fat shoots, short internodes
	1.0	TB12-Dalat	3.65^{cd}	4.03^{ab}	5.66^{ab}	Green, fat shoots, short internodes
	1.5	TB12-Dalat	3.53^{d}	4.08^{ab}	5.02^{bc}	Green, fat shoots, short internodes
	0	TB15-Dalat	2.17^{f}	3.64^{b}	3.37^{d}	Green shoots, purple stems
	0.5	TB15-Dalat	3.78^{bc}	4.32^{ab}	7.04^{a}	Green shoots, fat purple stems, short internodes
	1.0	TB15-Dalat	3.90^{b}	3.67^{b}	7.03^{a}	Green shoots, fat purple stems, short internodes
	1.5	TB15-Dalat	3.45^{d}	3.53^{b}	6.65^{a}	Green shoots, fat purple stems, short internodes
	0	TB16-Dalat	2.48^{e}	4.04^{ab}	3.37^{d}	Green shoots, purple stems
	0.5	TB16-Dalat	4.43^{a}	5.06^{a}	7.05^{a}	Green shoots, fat purple stems, long internodes
	1.0	TB16-Dalat	3.91^{b}	4.05^{ab}	6.09^{ab}	Green shoots, fat purple stems, long internodes
	1.5	TB16-Dalat	3.46^{d}	4.07^{ab}	5.67^{ab}	Green shoots, fat purple stems, long internodes

*Different letters (a, b, c⋯) in the same column indicate significantly different means using Duncan's test ($\alpha = 0.05$). PGRs: Plant growth regulators, BA: 6-benzyl amino purine and NAA: Naphthaleneacetic acid

was a result of different explant origins. In our study, explants derived from the apical shoot were used, while[14], used shoots from seedlings. The shoot growth also showed significant morphological differences. Therefore, this study evaluated the growth cycle, compared the morphology and used ITS sequences to identify three different morphological forms of *Dendrobium* spp., meanwhile, an *in vitro* propagation procedure for rapid conservation of these orchids has also been established.

CONCLUSION

The research results based on the phenotypic analysis and molecular identification showed that the regions of three variants of *D. anosmum* collected in the Di Linh district (Lam Dong) had homogeneous ITS region sequences and they had genetic relation close to the group *Dendrobium* aff. *anosmum*, which has similar morphological characteristics to *D. anosmum* rather than to *D. anosmum*. *In vitro* culture study showed that young shoots of white flower plants gave the highest regeneration rate of 80%, the orchid with light pink flowers showed 60% regeneration rate and plants with dark pink flowers showed the lowest regeneration rate 40%. *In vitro* plant lets grew well on SH medium supplemented with 30 g L^{-1} sucrose, 0.5 mg L^{-1} NAA, 1.0 mg L^{-1} BA, 9.0 g L^{-1} agar, 20% coconut water, 1.0 g L^{-1} activated charcoal. *In vitro* growth and development of young shoots still showed significant morphological differences.

SIGNIFICANCE STATEMENT

This study was conducted to evaluate the growth cycle, blooming season and structure of flowers as well as using ITS sequences to determine three different morphological forms. Besides, *in vitro* rapid multiplication was also studied on these 3 orchids, which was also done for conservation purposes. The results of the study were accurate in identifying species names based on morphology as well as using the molecular identification method. The effect of culture medium on shoot regeneration as well as the BA and NAA concentrations on the growth and development of shoots shown in the study were the basis for the rapid multiplication of three different morphological forms.

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