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Identification of Vietnamese Native *Dendrobium* Species Based on Ribosomal DNA Internal Transcribed Spacer Sequence

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Abstract

This study aimed to identify plant sources of Vietnamese *Dendrobium* from different regions based on sequences of the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA. We constructed an ITS1-5.8S-ITS2 sequence database of 32 Vietnamese native *Dendrobium* species. Comparison between sequencings of 32 Vietnamese native *Dendrobium* and *Dendrobium* species in the world by using ITS sequences, we have determined exactly the same pattern 23/32 of *Dendrobium* species. In the remaining 9 Vietnamese native *Dendrobium* varieties, 4 varieties were re-edited Latin names such as Hoang Thao tram Trang, D15 (Hoang Thao Tam Dao), D24 (Hoang Thao Vay rong la nho), and D25 (Hoang Thao Vay rong la trung); 5 samples have not been pinpointed as the same form including D11 (Hoang Thao Xoan), D12 (Hoang Thao Kieu tim), D14 (Hoang Thao Kieu trang), D19 (Hoang Thao Kieu trang Dong Nai), and D26 (Hoang Thao Thuy tien mo ga) species.

Keywords: *Dendrobium species, Orchidaceae*, Internal Transcribed Spacer (ITS)

1. Introduction

The genus *Dendrobium* is one of the largest genera in the Orchidaceae with over 1148 worldwide species. The enormous morphological diversification has hindered the establishment of consistent classification systems covering all major groups of this genus which was the second orchids after bulbophyllum orchid *(Bulbophyllum*) [1]. Many orchids are valuable herbs, traditionally applied to cure some problem of fertility and virility; their importance in treating nervous, cardiac, dermal, respiratory, and digestive disorders [2]. Southeast Asia can be considered as the homeland of *dendrobium* with hundreds of species, particularly in Vietnam, there are more than 100 species [3], and they are widely distributed across the regions of the country. The advantages of molecular techniques are capable to identify the diversity at molecular level, providing the basis for assessing the conservation value of species, identification of cultivars, selection of parents for breeding, and conservation of genetic resources such as: Restriction Fragment Length Polymorphism (RFLP); Amplified Fragment Length Polymorphism (AFLP); Random Amplified Polymorphic DNA (RAPD); Microsatellite or Simple Sequence Repeats (SSR); Inter-simple sequence repeats (ISSRs); and Internal transcribed spacer (ITS) sequencing. The molecular markers have been widely used for the identification of numberous orchids species: *Dendro-*

bium [4], *Cymbidium* [5], *Vanda* [6], *Paphiopedilum* [7], *Phalaenopsis* [8]. Particularly, ITS technique has been frequently used to identify Orchidaceae using biological molecular markers [9].

In this study, we constructed a sequence database of the internal transcribed spacer ITS1- 5.8S-ITS2 region for Vietnamese native *Dendrobium* species, which were identified accurately based on the morphological characters of vegetative and reproductive organs. The database covers most of the *Dendrobium* species from Vietnam and adjacent regions. The sequence readout of ITS1 and ITS2 regions of nuclear ribosomal DNA appeared with sufficient variation to discriminate *Dendrobium* at the interspecific level. In this study, ITS DNA sequencing was applied to identify Vietnamese native *Dendrobium* species samples obtained in different province of the country.

2. Materials and Methods

Many *Dendrobium* species have synonyms (different scientific names used for a single species). In this study, 32 samples of Vietnamese native *Dendrobium* (aged 2-3 years) species were collected in different provinces and grew at the Agricultural Genetics Institute. Genomic DNA extraction and PCR amplification were performed following the method of Trung et al [7]

Sequencing: The purified PCR products were sequenced directly by an ABI PRISM^{TM} 310 Genetic Analyzer (Applied Biosystems). The primers ITS1 and ITS4 were used for the sequence reaction. The ITS region of each individual was sequenced in the both 5' direction and 3'direction at least twice to avoid mutation introduced by Taq polymerase. The boundaries of the ITS1 and ITS4 were determined by comparing them with the sequences of the Orchids family species in the GeneBank.

Statistical analysis: The sequences were aligned and compared using the Clustal W programs and analyzed using the MEGA 5.2.1 programs.

3. Results and Discussion

3.1. Molecular maker to identify *Dendrobium* species based on ITS region sequences

By using ITS1 and ITS4 primer pairs, we amplified successfully ITS region by PCR products. These results were high quality with appreance of the only one band in size from 700-800 bp (Fig.1). These results were also consisted with the findings of other authors to amplify the ITS region on *Dendrobium* species [9]. The bands in size were clear, correct size should be able to use sequencing.

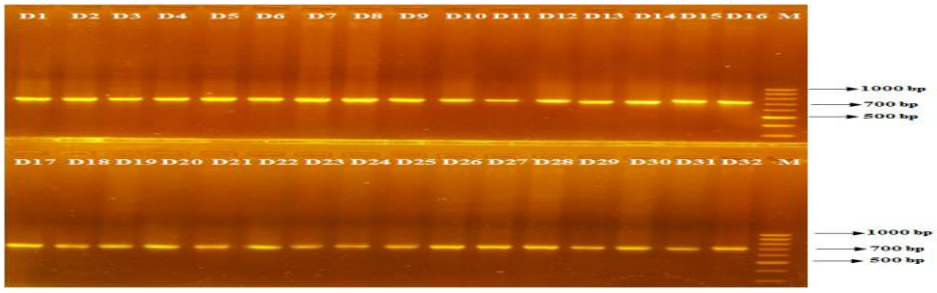

Figure 1. Electrophoresis of amplification ITS segment on 32 *Dendrobium* species by PCR with ITS1 and ITS4 primer pairs; *Lanes: 1-32: Dendrobium samples; M: 100bp ladder*

3.2. Analysis of 32 *Dendrobium* species by sequences

The results of sequencing showed that the 32 *Dendrobium* species were amplified and sequenced the ITS region include partial 18S region, entire regions ITS1, 5.8S, ITS2 and partial 28S area, total length of nucleotides obtained from 652 to 715 nucleotides with average 685.3 nucleotides. The percent of nucleotide as T (U) = 22.4%; C = 24.3%; A = 23.7%; and G = 29.6%. This result is consistent with the previous reports of Xu *et al*. [10] (2005).

Alignment on Vietnamese native Dendeobium and Dendrobium species in the world

To compare the difference of Vietnamese native *Dendrobium* species and *Dendrobium* species in the world, we were conducted the analysis of the samples of Vietnamese species and the world based on the analysis coming aligned columns. In this study, some results have been compared with the ITS sequences of some orchids form Vietnamese native *Dendrobium* species and the world based on GeneBank.

**** Alignment of D2 (Hoang Thao Chuoi ngoc Dien Bien) and D18 (Hoang Thao Chuoi ngoc) in GeneBank***

Through the analysis aligns upcoming column (alignments), ITS sequences of D2, D18 Vietnamese species with two species of *D.findlayanum* |KF143462.1|, |HQ114257.1| in the world, these results showed that in some statistical tables nucleotides 4 ITS sequences of species with fluctuations *D.findlayanum* number of nucleotide sequences from 688-694 nucleotides when it was compared with the sample sequence shows the two samples *D.findlayanum* D2 and D18 had 19 different nucleotides. When comparing the two samples D2 and D18 with two reference samples, the samples D2 and D18 have shown 8 different sequences than two samples of the world as *D. Findlayanum* species (Fig. 2). This result showed that D2 and D18 species did not much differ from the world's *D.findlayanum* species.

Similarly, most of Vietnamese native *Denrobium* species were also analyzed by alignment with *Dendrobium* species of the world to determine the difference in the order as well as to identify species/sub-species based on the ITS regions.

However, the comparison with the GeneBank database in order to compare with the similar taxa based on the reference sequence. BLAST results could not conclude exactly the species. For instance, BLAST similarities coverage and high identity sequences (99%), could not reverse impairment species name. BLAST results revealed the most homologous sequences in the GeneBank. Therefore, to determine the correct species name which should be made further analyses and compared to their morphological data to record the relationship through phylogenetic tree based on ITS region sequences.

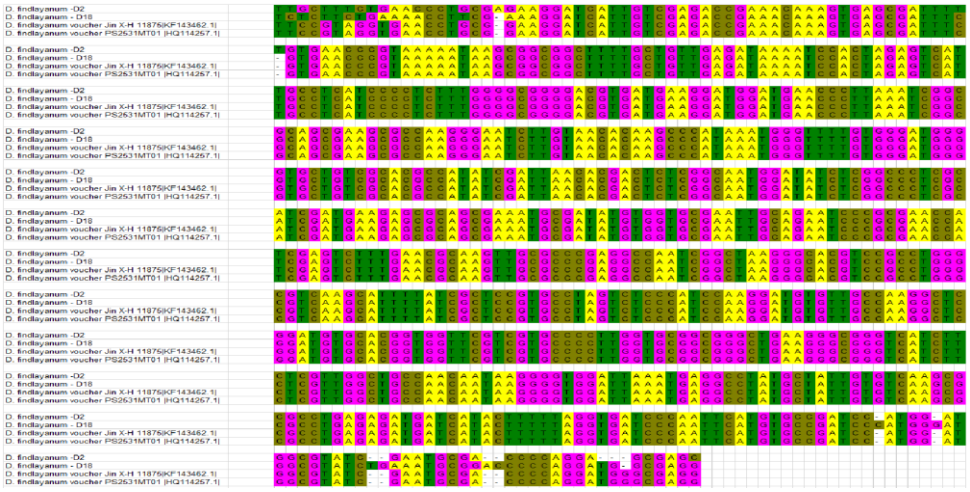

Figure 2. Alignment of D2 and D18 Vietnamese native *Dendrobium* sequences with two sequences of acession number *D. findlayanum* |KF143462.1|,|HQ114257.1|_species

3. 3. Phylogenetic trees base on ITS region sequences

According the phylogenetic trees, 32Vietnamese native *Dendrobium* species trees were divided into 17 different subgroups (Fig 3):

Group I included 3 Vietnamese species such as D4 (*D. anosmum*), D5 (*D.parishi.var alba*) and D6 (*D.parishi*); and 6 sequences with accession number HM590378.1, HM054736.1, HM054735.1, AB5936930.1 species on *D.parishi*, and 2 sequences on *D.asosmum* species with accession number EU477499.1, JN388570.1. In early diverging positions, D6 species was identified as *D. parishi* morphological but located near accession number EU47749.1, which was identified as *D. anosmum*, whereas 4 species with accession numbers included HM590378.1, HM054736.1, HM054735.1, and AB5936930.1 as *D. parishi* were located together in subgroups. This could be confirmed that the accession number EU47749.1 was *D. parishi* species, not anosmum, from which we deduced that D6 species was similar to *D.parishi* species. In late position divergence, D4, D5 and JN388570.1 samples were located in same subgroup. In terms of morphology, D4 sample was identified as *anosmum* species and D5 was identified as *D parishi.var alba.* However, two species including D4 and D5 were located with

accession number JN388570.1 subgroup on the identification percentage of 99%. Therefore, D5 sample should be determined as *D. anosmum.var alba*. Our observation agreed with the study of Dung *et al*. (2012), when they were analyzed two Vietnamese species as Hoang Thao Tram rung (*D. parishi*) and Hoang Thao Phi diep (*D. anosmum*) (Dung *et al*., 2012).

Group II included D9, D29 species and 3 accession numbers KF14399.1, AB593641.1, AB59352.1 as *D.primulinum* species was located in subgroup. D9 and D29 species were same divided into accession number AB59352.1 in subgroups at 99% bootstrap index. D9 and D29 species were distributed in both Northern and Central highlands. Morphologically, they have similar characteristics and identified as *D.primulinum* species. Therefore, D9 and D29 species were accurately identified as *D.primulinum* species.

Group III included D23 species which was identified as *D. aphylum* morphology and it was same located in subgroups with 4 accession numbers HM590384.1, HQ114247.1, HQ114248.1 and KF143430.1 at 100% bootstrap index. Therefore, D23 species was identified as Hoang Thao Hac vi (*D. aphylum*).

Group IV: D11 and D28 species were located in same group with 6 species in the world. In this group, D11 (*D.tortile*) divided into 4 different accession numbers EU4775071 (*D.nobile*), EU477507.1 (*D.fiedricksianum*), KF143518.1 (*D.sp.Jin XHs.n .9*) and EU477511.1 (*D.tortile*) in subgroup with bootstrap index up to 98%. In terms of morphology, D11 was different morphology as stems, leaves, flowers compared to accession number EU477507.1 (*D.fiedricksianum*), KF143518.1 (D. Sp.Jin XHs.n.9). From the results of the comparison on Blast, these templates are also messy unfocused. Therefore, we could not recognize the D11 sample as *D.tortile* or not. However, the D28 sample was located with accession numbers as KC205193.1 and JN38579.1 at 85% bootstrap index. Therefore, this indicated that D28 sample was identified as *D. nobile.*

Group V had three species including D27, D2 and D18 divided into two subgroups: Subgroup 1 included D27 (*D.aduncum*) species and divided into one group with 3 different species sequences as accession numbers JF713083.1, KF143428.1 and JN388580.1 at 99% bootstrap index. On other hand, the characteristic of the morphology on D27 was similar with the 3 *Dendrobium* species. Therefore, D27 sample should be identifying as *D. aduncum;* Subgroup 2 was included D2 and D18 samples, which were collected in two provinces. In terms of their form, it was exactly the same on stems, leaves, and flowers differs only Long tu Bac (D2) in the middle of the flower lip does not have spots. When we comparing sequences of them, it have only the bootstrap of 100% with 3 accession numbers as KF143246.1, HQ114257.1, and JN388589.1 belong *D.findlayamum* species. Therefore, D2 and D18 samples were identified as *D.findlayamum* species.

Group VI included D7 and D8 samples, these species were identified as *D.chrysanthum* and located in 6 different *D.chrysanthum* species in the world as the controls. D8 sample was identity at 100% when it was compared with two accession numbers of *D.chrysanthum* as JN388584.1 and FJ384738.1. Therefore, we concluded that D7 and D8 were identified as *D.chrysanthum* species.

Group VII included D30 (Hoang Thao Moi to- *D.brymerianum)* species, and 4 species as *D.brymerianum*: KF143432.1, HQ114233.1, JN388581.1 and EU477500. 1 in subgroups at 80% bootstrap index. D30 species was grouped with 44 species as *D.brymerianum* but relatively large genetic distance with the pattern of the world.

Group VIII included D3 (Hoang Thao Thai Binh - *D.moschatum*) species which was identified as *D. moschatum* (KF143492.1) at 100% with bootstrap index. However, in this group there was one form as *D.puchellum* (KF1435031.1) similar to *D.moschatum* (KF143492.1). In terms of morphology, these were identical on stems, leaves and flowers. However sample (KF1435031.1) was identified as *D.puchellum* in Latin name. In terms of morphology, D3 was determined as *D.moschatum*, thereby reconfirming that the D3 sample was corrected as *D.moschatum* species based on the sequencing the ITS region.

Group IX included 2 Vietnamese species such as D1 (HT Long Long nhan Dien Bien), D15 (HT Tam Dao) and 3 species of world as *D. fimbriatum* classification. In this group, the sample D1 (*D. fimbriatum*) and D15 (*D.daoense*) were shown identity from 86-100% bootstrap index with 3 species of *D. fimbriatum* with accession numbers HQ114229.1, JN388588.1, KF143461.1. However, morphological identification on D15 species was identified as *D.daoense*. Therefore, we confirmed that D15 species was collected as *D. fimbriatum*, which was not as *D.daoense* species.

Group X included 3 Vietnamese species as D16, D20, D21 and 4 species as *D. chynsotoxum* with accession numbers HQ114221.1, JN388585.1, HQ114223.1, EU477501.1 and 1 sample of species D.cappillies (HM590379.1) In the 5 samples of the world, HM590379.1 sample sequencing was wrong because if this form is mandatory *D.cappillies* species it must be located on the group XII, could not locate into this grouped as sequences belong to *D. chynsotoxum*. The D16, D20 and D21 were divided into two subgroups consisting D16 (Hoang Thao Hoang Hoang lap Tay Bac) into one group where located with 4 species as *D. chynsotoxum*. Other subgroups included D20 (Hoang Thao tieu Hoang lap), D21 (Hoang Thao Dai Hoang lap) and accession numbers EU477501.1 with high bootstrap index (98%). In terms of mophology, the form Hoang Thao Hoang lap species were shape, leaf shape and color of flowers marking the same, they are only about the size of a little flower and distributed in different regions. Thereby may notice that, 3 samples as D16, D20 and D21 species have the genetic separation, but they were same species *D. chynsotoxum*.

Group XI included 5 species as D10 (*D. hancokii*) and 4 species *D. hancokii* sequences in a group with the 76-88% bootstrap index. However, D10 sample was separated genetic distance is quite far with 4 *D. hancokii* species of the world.

Group XII included D17 (*D.capillipes*) and 4 *D.capillipes* species: AF362035.1, KF143433.1, JN388582.1, HQ114224.1, and they divided into two branches with D17 sample and accession numbers AF362035.1 with bootstrap index (88%) and the 3 samples were located together form a branch. In forms, D17 (Hoang Thao Kim Diep) sample was recognized as *D.capillipes*, thereby confirming that the corrected form is *D.capillipes* D17 after sequencing on the ITS region.
Group XIII included 7 samples which had 5 Vietnamese samples such as D12 (*D.amabile*), D13 (*D.thyrsiflorum*), D14 (*D.farmeri*), D19 (*D.farmeri*), D26 (*D.haveyanum*) and two accession numbers KF143519.1, KC205200.1 of *D.thyrsiflorum*, and they divided into subgroups. Subgroup 1 had 5 samples and accession number KC205200.1 with bootstrap index at 100%. D12 and D13 samples were separated within the same group with 91% bootstrap index, while D14, D19 and D26 have the same one branch with KC205200.1 samples with 100% bootstrap index. In terms of form, Hoang Thao Kieu varieties as D12, D13, D14, D19 and Thuy Tien Hoang Thao mo ga (D26) are very similar in morphology, such as stems, leaves, they differ only in the color of flowers and region distribution. However, in the phylogenetic tree showed that the samples collected have very high bootstrap index from 96-100%, while the morphological identification Latin name is different. Therefore it could be concluded that the samples collected when no precision on morphological identification or they have extremely close genetic relationship to use ITS not enough to identify their exact species. Only could determine D13 (HT Kieu Vang) were recognized as *D.thyrsiflorum* species.

Group XIV included D24 and D25 (*D.lindleyi species*) species and 4 samples collected sequences as accession numbers KF143479.1, JN388595.1, KF143478.1 and HQ114251.1 (*D.jenkinsii species)* with bootstrap index from 94-100%. In terms of morphology, D24 and D25 species were easy to get wrong with *D.jenkinsii*. Appearance of *D.lindleyi* is often confusing with snake scales or fins, when comparing the flower stems and branches, stems larger scales scaly, flower scales have long stem from 5-15 flowers and *D.jenkinsii* species were less flowers than *D.lindleyi*. Therefore, the results of the ITS region sequencing and comparative phylogenetic tree showed that D24 (Hoang Thao Vay rong la nho) and D25 (Hoang Thao Vay rong la trung) were *D.jenkinsii* species.

Group XV inculded Hoang Thao Nhat diem hong (*D.draconis* Rchb.f - *D31*) with 5 species in accession numbers as HM054628.1, EU477503.1, JF713101.1 (*D. draconis)* with bootstrap index 100%. Therefore, D31 was indicated as *D. draconis* species.

Group XVI included D22 (Hoang Thao Bach hac langbiang- *D.wattii*) and 3 other varieties such as KF143525.1, KF143481.1, KF143485.1. In this group, the D22 was into branch

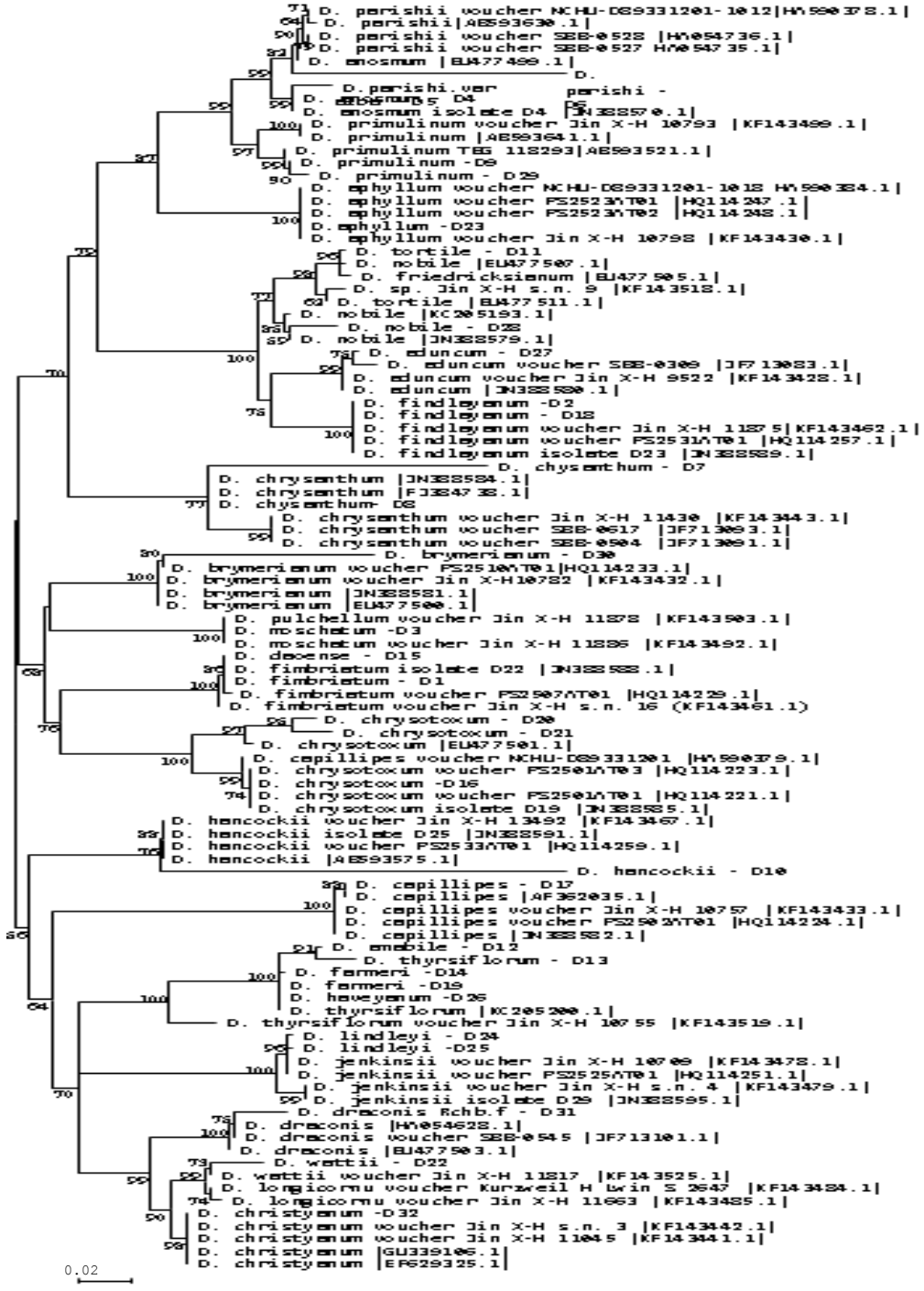

Figure 3. Phylogenetic trees based on ITS sequences

with *Dendrobium voucher Jin XH 11 817* (KF143525.1) at 73% bootstrap index. But *D. wattii* group has very close with *D.longicomu* (KF143481.1, KF143485.1) with bootstrap index from 74-99%. In terms of morphology, *D. wattii* and *D.longicomu* have similar morphology so easy to confuse the distinction.

Therefore, D22 Hoang Thao Bach hac langbiang confirmed exactly *D.wattii* species

Group XVII included Hoang Thao Dai bach hac (*D. christyanum* -D32) and 4 species with accession numbers F143442.1, KF143441, GU339106.1, and EF629325.1. These varieties were identified *D. christyanum* species with bootstrap index at 99%. It suggests that, the D32 sample was identified as *D. christyanum* species.

Table 1: Identification on Vietnamese native *Dendrobium* species based on ITS sequences

Symbols	**Vietnamese names**	**Morphological**	**Identification based on ITS sequences**
D1	Hoang Thao Long nhan Lai Chau	*D. fimbriatum*	*D. fimbriatum*
D2	Hoang Thao Chuoi ngoc Dien Bien	*D. findlayanum*	*D. findlayanum*
D3	Hoang Thao Thai Binh	*D. moschatum*	*D. moschatum*
D4	Hoang Thao Phi Diep tim	*D. anosmum*	*D. anosmum*
D5	Hoang Thao Tram tim	*D.parishii*	*D.parishii*
D6	Hoang Thao Tram trang	*D.parishi .var alba*	*D.anosmum.var alba*
D7	Hoang Thao Ngoc Van Vang	*D. chrysanthum*	*D. chrysanthum*
D8	Hoang Thao Phi Diep vang	*D. chrysanthum*	*D. chrysanthum*
D9	Hoang Thao Long tu Bac	*D. primulinum*	*D. primulinum*
D10	Hoang Thao Truc	*D. hancockii*	*D. hancockii*
D11	Hoang Thao Xoan	*D. tortile*	Not indentify
D12	Hoang Thao Kieu tim	*D. amabile*	Not indentify
D13	Hoang Thao Kieu vang	*D. thyrsiflorum*	*D. thyrsiflorum*
D14	Hoang Thao Kieu trang	*D. farmeri*	Not indentify
D15	Hoang Thao Tam Dao	*D. daoense*	*D. fimbriatum*
D16	Hoang Thao Hoang lap Tay Bac	*D. chrysotoxum*	*D. chrysotoxum*
D17	Hoang Thao Kim Diep	*D. capillipes*	*D. capillipes*
D18	Hoang Thao Chuoi Ngoc	*D. findlayanum*	*D. findlayanum*
D19	Hoang Thao Kieu Trang Dong Nai	*D. farmeri*	Not indentify
D20	Hoang Thao Tieu Hoang lap	*D. chrysotoxum*	*D. chrysotoxum*
D21	Hoang Thao Dai Hoang lap	*D. chrysotoxum*	*D. chrysotoxum*
D22	Hoang Thao Bach hac langbiang	*D. wattii*	*D. wattii*
D23	Hoang Thao Hac vi	*D. aphylum*	*D. aphylum*
D24	Hoang Thao Vay rong la nho	*D. lindleyi*	*D. jenkinsii*
D25	Hoang Thao Vay rong la trung	*D. lindleyi*	*D. jenkinsii*
D26	Hoang Thao Thuy tien mo ga	*D. haveyanum*	Not indentify
D27	Hoang Thao Vani	*D. aduncum*	*D. aduncum*
D28	Hoang Thao Dui ga	*D. nobile*	*D. nobile*
D29	Hoang Thao long tu da	*D. primulinum*	*D. primulinum*
D30	Hoang Thao Moi to	*D.brymerianum*	*D.brymerianum*
D31	Hoang Thao Nhat diem hong	*D. draconis Rchb.f*	*D. draconis Rchb.f*
D32	Hoang Thao Dai bac hac	*D. christyanum*	*D. christyanum*

The information through the ITS region sequencing showed that the majority of samples collected during the study was identifying compatible with morphological identification. 23/32 species were identified by recognition molecule forms such as D1, D2, D3, D4, D5, D7, D8, D9, D10, D13, D16, D17, D18, D20, D21, D22, D23, D27, D28, D29, D30, D31, and D32 species (Table 1). However, during the study, the varieties were identified based on morphological form, then sequencing the ITS region. Comparison on sequencing between 32 Vietnamese native Dendrobium orchids and sequencing of *Dendrobium* orchids varieties in the world, we have determined exactly the same pattern 23/32 of *Dendrobium* varieties. In the remaining 9 Vietnamese native *Dendrobium* species, and 4 species were edited form Latin names such as (Hoang Thao tram Trang), D15 (Hoang Thao Tam Dao), D24 (Hoang Thao Vay rong la nho), and D25 (Hoang Thao Vay rong la trung); 5 samples have not been pinpointed as the same form D11 (Hoang Thao Xoan), D12 (Hoang Thao Kieu tim), D14 (Hoang Thao Kieu trang), D19 (Hoang Thao Kieu trang Dong Nai), and D26 (Hoang Thao Thuy tien mo ga).

Explanations for this phenomenon has many different causes, such as collecting seed samples confusion about different Latin name; The process of preservation can breed confusion or data on GeneBank insufficient information with the same sample by the Latin name and dissimilar ITS region (Table 1).

In summary, although the species is still limited but the ITS sequences again showed they promote strength is a universal DNA barcode in the delimitation of species and subspecies level for plant group flowering. The result showed that the majority of varieties were identified their name and species. Some varieties were different in name by morphological and ITS sequence region. Through the analysis showed that the location of species used in the phylogenetic tree based on sequence analysis of the ITS which helps confirm correct scientific names of varieties. The success of this study was make important prerequisite for us to expand the sample size like (the same sample in the species, *Dendrobium* species, or more species in Orchideacea), or selection some molecular marker (16 rRNA genes, gene matK ...) to construct DNA barcodes for valuable orchids in Vietnam.

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