

# Molecular Phylogeny of rDNA-ITS on Native *Dendrobium* in Lampung

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## Abstract

*Dendrobium*, a flagship collection of the Liwa Botanical Garden, is an endemic flora of the Southern Sumatra that requires preservation. One of the challenges in its conservation and potential development is the molecular identification. This molecular identification utilizes DNA barcoding with the rDNA-ITS marker as a practical, rapid, accurate, effective, and efficient alternative, complementing previous species-level identification results based on morphological characteristics. Amplification results from 5 selected samples showed specific bands measuring 300 bp. Sequence data analysis using BioEdit and MEGA V.11.0.11 software with 1000 bootstraps grouped all accessions into the same main cluster with a similarity range of 94–100%. Phylogenetic analysis revealed that accession D2 is similar to *D. signatum* from Japan (AB593662.1), and accession D3 to *D. densiflorum* (HQ114255), D4 to *D. nobile* (LC011413.1), accession D6 to *D. trigonopus* (KF143730.1), and accession D12 to *D. faciferum* (LC192955.1) from China. The results of this study will enrich the taxonomic and phylogenetic data of *Dendrobium*, which is essential for conservation and serves as a foundation for its development as a medicinal herbal plant.

**Keywords:** molecular phylogeny, rDNA-ITS, *Dendrobium*, molecular identification, Lampung

## 1. INTRODUCTION

The genus *Dendrobium* includes more than 1,800 native species [1], which is widely distributed across Southeast Asia [2], including Indonesia [3] [4]. The development of the native *Dendrobium* potential in Lampung has been carried out by the Liwa Botanical Garden through exploration and collection activities, with 48 accession numbers [5]. Based on our previous studies [6]-[11], challenge in the conservation and development of native *Dendrobium* in Lampung is identification [12]. Identification has so far been done conventionally through the observation of morphological [13]-[15] and anatomical [16][17] characteristics. This method is considered limited, particularly due to the high intraspecific and interspecific variations in morphological traits such as shape and size of flowers, leaves, and stems between individuals within the same species [18]. Furthermore, environmental factors also influence morphological

expression, which leads to difficulties in distinguishing true genetic differences based on phenotypic variation [19][20]. Additionally, anatomical data are often insufficient to differentiate species with similar morphology [21] [22]. Therefore, alternative methods for more accurate, effective, and efficient identification of native *Dendrobium* in Lampung are required through molecular approaches.

A commonly used molecular approach for plant identification [23][24] is DNA barcoding with specific sequence markers from the nucleus and chloroplast [25]. One of the recommended universal barcode candidates by The Consortium for the Barcode of Life (CBOL) is ribosomal DNA Internal Transcribed Spacer (rDNA-ITS), in addition to maturase-K (matK) and ribulose-1,5-bisphosphate carboxylase oxygenase large subunit (rbcL) [26]. Perwitasari et al. reported DNA barcoding identification results for the medicinal orchid *D. discolor* Lindl. Tanimbar, which showed that the ITS marker of 1500 bp is species-specific and capable of distinguishing species levels [27]. Perwitasari et al. [27] and Su'udi et al. [28] demonstrated that this marker has higher genetic diversity with low homology, making it a potential recommendation for molecular identification. Liu et al. reported the results of rDNA-ITS sequence analysis, which showed the authentication and differentiation capabilities of *Dendrobium* species, supporting its use in molecular identification [29]. Zhu et al. also used the ITS2 marker to identify *D.*

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**Figure 1.** Leaf samples collection of native *Dendrobium* in Lampung of (a) D2, (b) D3, (c) D4, (d) D6, and (e) D12.

*officinale*, proving its effectiveness in differentiating this species from others [30].

These studies have shown that molecular approaches using DNA barcoding with rDNA-ITS markers have proven effective in overcoming the limitations of identification based on morphological and anatomical characters. Raskoti and Ale explains that this is due to the high variability of rDNA-ITS between species [31], while being conserved within the same species, thus allowing for more accurate identification [32]. This study aims to complement the sustainability of our previous research, namely to develop a molecular approach for the identification of native *Dendrobium* species from Lampung down to the species level [5][12]. The results of this study will also enrich the taxonomic and phylogenetic data, which are essential for the conservation and sustainable utilization of biodiversity, while also serving as the basis for the development of its potential as a herbal medicinal plant.

## 2. MATERIALS AND METHODS

### 2.1. Materials

A survey and collection of native *Dendrobium* samples in Lampung were conducted at the greenhouse of the Liwa Botanical Garden. The collected samples were leaves, and the collection process followed the methods described in the previous work [33]. The collection process began with selecting healthy and representative leaves from each *Dendrobium* species to ensure that the obtained samples could represent genetic variation. The samples were stored and further analyzed in the laboratory. Environmental factors such as altitude,

temperature, humidity, and soil type were also carefully monitored and recorded during the sampling process.

### 2.2. Methods

#### 2.2.1. DNA Genom Isolation, Qualification, and Quantification

The DNA isolation was performed using the cetyl-trimethyl-ammonium bromide (CTAB) method following the protocol of Windiyani et al. [34]. The DNA isolation process consists of five stages: cell isolation, cell wall and membrane lysis, extraction into solution, purification, and precipitation. The final result is a DNA pellet dissolved in 40  $\mu$ L Tris-EDTA (TE) buffer with a pH of 8.0 and stored in a freezer at -20  $^{\circ}$ C until the DNA is used [35]. To assess DNA purity, two tests were conducted: quantity test using a spectrophotometer and quality test using electrophoresis. The DNA purity level is determined by dividing the absorbance value at 260 nm by that at 280 nm. DNA quality was tested using 1% agarose gel electrophoresis at 60 V for 105 min [36].

#### 2.2.2. Ampfication DNA

The initial preparation step for amplification involved the creation of a PCR cocktail mix with a total volume of 20  $\mu$ L, consisting of 16  $\mu$ L ddH<sub>2</sub>O, 1  $\mu$ L forward primer, 1  $\mu$ L reverse primer, and 2  $\mu$ L DNA template. The universal primer sequences used were rDNA-ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and rDNA-ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [28]. DNA amplification followed the method described

in the previous works [26][27][28], with 35 cycles at the following temperatures: denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 90 sec, and a post elongation step at 72 °C for 5 min. The quality of the amplified DNA fragments was assessed using 2% agarose gel electrophoresis at 60 V for 105 min with an electrode distance of 19.4 cm. PCR products were visualized using a Gel Doc UV system, and sequencing was carried out using the Sanger dideoxy method [23].

### 2.2.3. Data Analysis

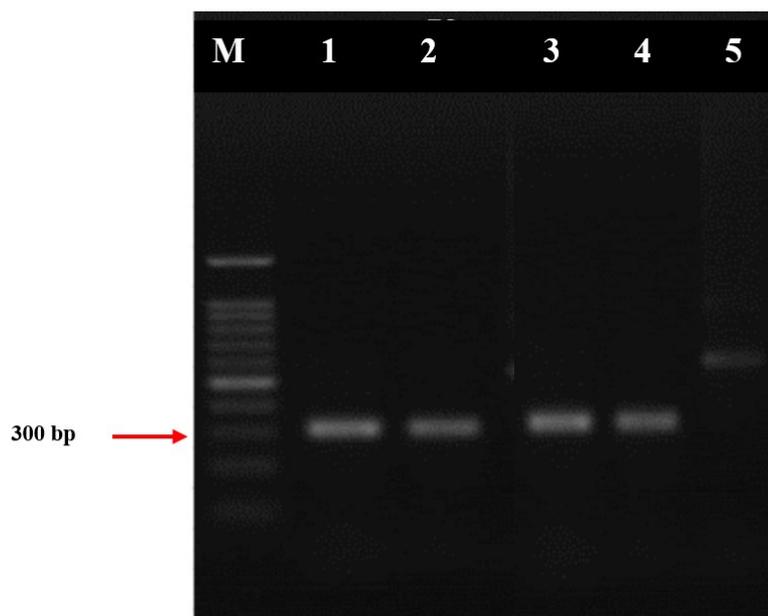
Sequencing results were analyzed using Sequence Scanner software, and nucleotide sequences were assembled with EditSeq and SeqMan from the DNASTAR Lasergene software suite. Homology searches were conducted using BLAST against the DDBJ and NCBI databases to compare sequences from different isolates. Sequence alignment was performed using Clustal W in BioEdit, and phylogenetic reconstruction was done with MEGA V.11.0.11 using the Neighbour Joining method and the Kimura-2 parameters. Statistical analysis of the internal branches was performed with bootstrap values using 1,000 replications to assess the reliability of the phylogenetic tree [11][37].

## 3. RESULTS AND DISCUSSIONS

### 3.1. Sample Collection

The samples used in the molecular identification with DNA barcoding using rDNA-ITS markers were selected based on the results of leaf morphology analysis, which is published separately. The samples used are 5 accessions, namely D2, D3, D4, D6, and D12, representing each cluster (Figure 1). The selection of this small sample size was made for the initial purpose of genetic exploration, considering the limitations of available resources and time. However, the selected accessions represent different morphological variations and encompass various locations in Lampung, providing a useful preliminary overview of the genetic relationships among species.

The data show that all samples were taken from various locations with geographical differences and environmental factors such as altitude, temperature, humidity, and soil type, which influence leaf morphology. D2, D3, D4, and D6 originates from HL. Reg. 49 B, Reg. 44 B, Reg. 43 B, and Reg. 45 B, Sugarcane Plantation in Sumber Jaya, West Lampung, respectively, while D12 originates from Mount Pesagi, Hujung Village, Belalau District. Genetic variation in *Dendrobium* is often affected by differences in altitude, which in turn influence temperature and humidity and ultimately affect



**Figure 2.** The results of DNA barcoding amplification using the ITS1 and ITS4 markers. M = 100 bp DNA Marker, 1 = D2, 2 = D3, 3 = D4, 4 = D6, and 5 = D12.

**Table 1.** The isolates with the highest similarity to the native *Dendrobium* in Lampung.

Sample Code	NCBI			
	Identification	Similarities	Acc. Number	Origin
D2	<i>D. signatum</i>	100%	AB593662.1	Japan
D3	<i>D. densiflorum</i>	99%	HQ114255	China
D4	<i>D. nobile</i>	94%	LC011413.1	China
D6	<i>D. trigonopus</i>	97%	KF143730.1	China
D12	<i>D. faciferum</i>	96%	LC192955.1	China

genetic expression and species adaptation to their environment [38][39]. Orchids growing in drier areas or higher temperatures tend to have smaller or thicker leaves as an adaptation to environmental stress. Additionally, soil type plays a role in determining nutrient availability, which can affect species growth and development, as it is linked to observed genetic variation. The interaction of these environmental factors in ecological dynamics influences genetic expression related to protein synthesis involved in stress responses, which in turn shapes the genetic diversity in *Dendrobium* species. Wang et al. [38] explains that more specific locations, such as tropical climates or highland areas, can significantly affect leaf characteristics. The variation in leaf shape based on the origin location represents a strong environmental or genetic influence on leaf morphology [39].

Previously, Liu et al. [39] compared the genetic diversity of *Dendrobium* from various regions in China, which showed significant genetic variation among populations from different ecological conditions, such as differences in altitude and climate. Similarly, Wang et al. [38] compared *Dendrobium* species from various Asian countries, revealing clear patterns of genetic variation that can be linked to environmental variation and the ecological history of the species. By comparing the genetic diversity of *Dendrobium* from Lampung with other regions, this study can provide deeper insights into how geographical and ecological factors influence the genetic composition of the species.

### 3.2. DNA Qualification and Quantification

The DNA qualitative test results from the leaf sample extraction of *Dendrobium* using 1% electrophoresis gels provided a visualization of

intact DNA. Meanwhile, the quantitative DNA test results using UV-Vis spectrophotometry showed a purity range of 0.8–1.5, in accordance with the established standards. Bunu et al. explains that DNA purity is generally calculated with the 260/280 nm absorbance ratio, with values ranging between 1.8–2.0 [40]. The quantification values of the samples indicate protein contamination, but they are still suitable for further analysis.

### 3.3. Amplification of rDNA-ITS

The DNA amplification results with primers ITS1 and ITS4 showed a specific band of 300 bp (Figure 2). This indicates that these primers successfully amplified the DNA corresponding to the target. Mahfut et al. [25] also reported the amplification of 300 bp band using these primers for orchid species.

### 3.4. Molecular Characterization of rDNA-ITS

The results of the DNA sequence consensus analysis for 5 *Dendrobium* accessions D2, D3, D4, D6, and D12 showed nucleotide sequence lengths of 308, 321, 328, 305, and 325 bases, respectively. Based on the nucleotide sequence analysis, the highest GC content was found in accession D2 at 51.5%, followed by accessions of D3, D4, D6, and D12 with GC contents of 49.6%, 37.4%, 31.8%, and 31%, respectively. Bowers et al. reported that variations in GC content significantly affect DNA stability and contribute to sequence divergence across different *Dendrobium* genotypes [41]. Wang et al. also explained that GC rich regions play a role in maintaining DNA integrity against genetic variability and mutations in *Dendrobium* [37]. The results of homologous sequence searches using the BLAST program on NCB to verify that the test samples are *Dendrobium* showed the highest

**Table 2.** Frequency of amino acid content on accession of native *Dendrobium* in Lampung.

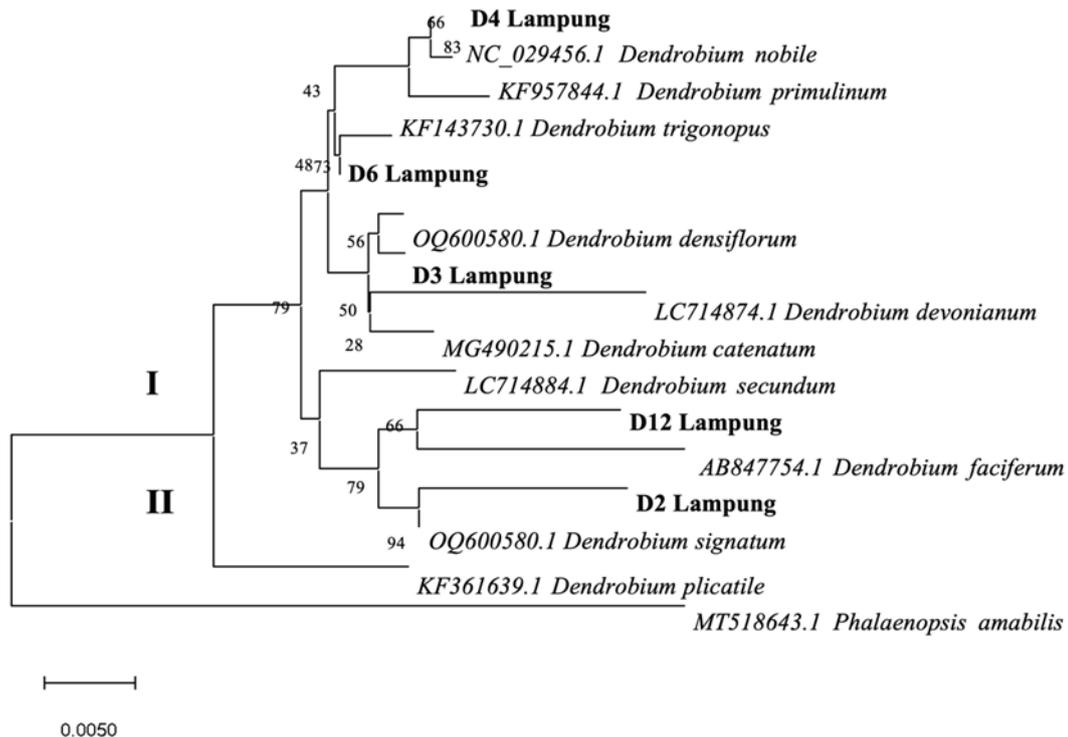
Sample	Frequency of amino acid (%)																			
	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr
D2	7.89	3.95	5.26	1.32	1.32	11.84	1.32	9.21	7.89	9.21	2.63	3.95	3.95	1.32	5.26	7.89	6.58	6.58	1.32	1.32
D3	4.30	1.08	8.60	3.23	1.08	6.45	6.45	8.60	5.38	12.90	1.08	4.30	2.15	0.00	13.98	8.60	2.15	4.30	1.08	4.30
D4	3.23	3.23	3.23	4.30	2.15	2.15	3.32	6.45	3.23	9.68	2.15	3.23	4.30	6.45	7.53	7.53	12.90	8.60	1.08	5.38
D6	3.45	5.17	1.72	1.72	6.90	1.72	0.00	10.34	5.17	15.52	3.45	1.72	3.45	5.17	6.90	10.34	10.34	1.72	0.00	5.17
D12	1.35	1.35	1.35	2.70	16.22	1.35	1.35	14.86	2.70	12.16	2.70	9.46	0.00	1.35	4.05	8.11	9.46	2.70	1.35	5.41

similarity index percentage in accession D2 at 100% with the *D. signatum* isolate from Japan (AB593662.1), while the lowest was in accession D12 at 96% with the *D. faciferum* isolate from China (Table 1).

A total of 80% from all accessions closely resemble isolates from China. This finding suggests that gene flow or historical trade might have contributed to the close genetic relationship between the two populations. This could be due to plant breeding activities and conservation biology related to the orchid trade from China [42]. Trade and plant distribution can lead to genetic exchange between geographically separated populations [39]. This suggests that, the history of trade also has the potential to influence plant genetic diversity in addition to ecological factors. The results of nucleotide alignment analysis using the ClustalW program in MEGA software revealed nucleotide differences between *Dendrobium* and 5 other isolates, caused by mutations in accessions D2, D3, D4, D6, and D12. The alignment results of the two accessions showed point mutations in the form of deletions, insertions, transitions, and transversions. These mutations led to changes in amino acids, affecting the amino acid frequency and genetic distance values in each accession. This indicates variations in genetic composition and protein expression [43].

The highest amino acid frequency in accession D2 was Gly (11.84%), Leu (9.21%), and Ile (9.21%). The highest amino acid frequency in accession in D3 was Arg (13.98%) and Leu (12.90%). The highest amino acid frequency in accession in D4 was Thr (12.90%) and Val (8.60%). The highest amino acid frequency in accession in D6 was Leu (15.52%). Meanwhile, the highest amino acid frequency in accession in D12 was Phe (16.22%) and Ile (14.86%) (Table 2). This indicates that each amino acid formed plays an important role in specific metabolic activities in tissues, expressed in the formation of protein structures and molecular stability. Katz et al. explains that the diversity of amino acid frequency patterns reflects genetic variation among accessions caused by differences in environmental conditions and natural genetic variation among populations [44].

The high-frequency distribution of Leu, Ile, and



**Figure 3.** The phylogenetic tree of native *Dendrobium* in Lampung using rDNA-ITS with 1000 bootstrap replicates.

Phe in most accessions suggests that these hydrophobic amino acids likely play a crucial role in protein structure in *Dendrobium*, related to genetic stability and specific protein functions [45]. Additionally, the presence of certain amino acids, such as Arg, Thr, and Val, in significant percentages is likely associated with specific protein functions involved in environmental responses or metabolism. Arg plays a crucial role in the synthesis of nitric oxide, a signaling molecule that regulates various physiological processes, including vasodilation and immune responses. Thr is vital for protein synthesis and contributes to maintaining immune function. Val essential for tissue repair, and energy production. The significant presence of these amino acids in proteins suggests their involvement in metabolic pathways and environmental response mechanisms [37][44].

The phylogenetic tree reconstruction, presented as a dendrogram, groups all accessions into two main clusters, I and II (Figure 3). All accessions are grouped within cluster I, while cluster II consists only of the isolate *D. plicatile* (KF361639.1) from China. The constructed phylogenetic tree shows that accession D2 has a very close relationship with *D. sigantum* from Japan (AB593662.1), accession

D3 is closely related to *D. densiflorum* from China (HQ114255), accession D4 is close to *D. nobile* from China (LC011413.1), accession D6 is close to *D. trigonopus* from China (KF143730.1), and accession D12 is close to *D. faciferum* (LC192955.1). The cluster patterns formed are divided based on accessions that share similarities in traits [26]. Differences within each cluster lead to the formation of subclusters, indicating genetic diversity within the species. However, the diversity among these species is still considered very close, as the phylogenetic tree only forms subclusters [25] [27].

The cluster pattern formed is divided based on accessions that have similar relationships based on the characters they share [26]. The differences within each cluster will form subclusters, indicating genetic diversity within the species. However, the diversity between these species is still considered very close because the phylogenetic tree only forms subclusters [27][28]. Bootstrap value indicates the level of confidence in each branch of the sub-cluster. Some branches have relatively high bootstrap values (> 70), such as the accessions D2 and D3 with bootstrap values of 94 and 79, respectively. Lemoine and Gascuel explained that

the larger the bootstrap value used, the higher the confidence in the topology of the reconstructed tree, which is greatly influenced by random effects based on the distribution of characters in the data [46]. A cluster is considered reliable if the bootstrap value is 90 [47].

The implications of this research can be used as baseline data for conservation efforts of *Dendrobium* in the Liwa Botanical Garden, where genetic variation analysis can be a focus of conservation to prevent the loss of genetic diversity. The phylogenetic data from DNA barcoding analysis using the rDNA-ITS marker provides valuable insights into the genetic diversity of *Dendrobium* species at the Liwa Botanical Garden, which is useful for conservation strategies. The data help us to identify populations with higher genetic diversity, which are more resilient to environmental pressures and diseases, and prioritizes the conservation of species or populations with higher genetic value. Additionally, phylogenetic data can identify species genetically related to endangered populations, supporting species recovery programs. Thus, this finding enhances the understanding of biodiversity and provides a strong foundation for genetic-based conservation decision-making.

#### 4. CONCLUSIONS

The molecular identification of DNA barcoding with the rDNA-ITS marker on 5 accessions of native *Dendrobium* from Lampung showed specific bands measuring 300 bp. The sequence data analysis grouped all accessions into the same main cluster with a highest similarity range of 94–100%. Phylogenetic analysis revealed that accession D2 is similar to *D. signatum* from Japan (AB593662.1), accession D3 closes to *D. densiflorum* from China (HQ114255), accession D4 closes to *D. nobile* from China (LC011413.1), accession D6 closes to *D. trigonopus* from China (KF143730.1), and accession D12 closes to *D. faciferum* from China (LC192955.1). The implications of this research can be used as baseline data for conservation efforts of *Dendrobium* in the Liwa Botanical Garden through the prevention of genetic diversity loss.

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##### Conflicts of Interest

The authors declare no conflict of interest.

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