Genetic relationship analysis of *Dendrobium anosmum* Lindl. var. semialba based on the chloroplast *matK* and *rbcL* genes

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ABSTRACT

Dendrobium anosmum Lindl. var. semialba has variants of flower shapes. Currently, it has high economic value and is favored on the market. In this study, the genetic relationship of *Dendrobium anosmum* Lindl. var. semialba species was determined based on the analysis of chloroplast *matK*, *rbcL* gene sequences. The *matK* and *rbcL* genes of twelve species were amplified and their DNA sequenced. These DNA sequences were compared, calculated genetic distance and constructed phylogenetic tree. The results showed that 100% of samples were amplified and sequenced successfully. The analysis of *matK* sequences showed that 12 species had very high genetic similarity with the low genetic distance of 0 - 0.001; the nucleotide sequences were almost unchanged except for one variable nucleotide position in TB1 and TB1 was in a separate branch of the phylogenetic tree. The analysis of *rbcL* sequences showed that all species had a low genetic distance of 0 - 0.012 and had 7 mutant positions in nucleotide sequences of TB1 and TB5. These species were in a separate branch of the phylogenetic tree while the other species were grouped in the other branch of the phylogenetic tree. The study provided a reliable molecular database of the *Dendrobium anosmum* Lindl. var. semialba for identification, classification, biodiversity assessment and conservation of genetic resources.

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1. Introduction

The orchid family (Orchidaceae) is one of the largest families of flowering plants in the world. There are 20,000 species in 850 genera in this family. *Dendrobium*, one of the largest genera in Orchidaceae, having more than 1,100 species identified, distributed in many parts of the world. Southeast Asia can be considered as the homeland of *Dendrobium* with hundreds of species, especially there are more than 100 species in Vietnam, widely distributed in all regions of the country (Hazlina et al., 2013; Xu et al., 2013; Tran et al., 2018). *Dendrobium anosmum* Lindl. is widely grown and has high economic value because of the thick leaves, arranging in 2 rows along the body and the beautiful flowers (Cao, 2018). In particular, the rare native *Dendrobium anosmum* Lindl. var. semialba, being from the natural forests of the Di Linh plateau Lam Dong province, have been cultivated domestically. It is very popular in the market today thanks to its characteristic aroma and unique variation of the flower.

Characteristically, *Dendrobium* species have a

wide geographical distribution and ability to produce a large number of hybrids with different morphologies. The high morphological diversity is one of the difficulties in taxonomy, especially the taxonomy based on morphology. The taxonomy of species is complex also because of the large distribution region (Xiaohua et al., 2009). Besides comparative vegetative anatomy and plant systematics, genetics has been used as a powerful tool for taxonomy and studying genetic relationships among *Dendrobium* species (Hazlina et al., 2013; Moudi et al., 2013a). Accurate genetic relationship information has been essential for the conservation of genetic resources, identification of plant varieties, selection of parents for propagation (Hazlina et al., 2013; Tran et al., 2018).

Currently, molecular techniques are widely used in the genetic analysis of orchids. The common molecular data used in plant systematics come from two sources: chloroplast DNA (cpDNA) and nuclear ribosomal DNA (nrDNA). Chloroplast DNA has been the most extensively used source of data in plant phylogenetic analysis. In particular, *matK* and *rbcL* are chloroplast genes having high effectiveness in genetic correlation analysis (Hollingsworth, et al., 2009; Moudi et al., 2013b; An et al., 2019). These makers have been successfully applied in many studies about genetic relationships and taxonomy of rice, Apocynaceae, Acacia, Orchidaceae, Araliaceae, etc. (Cabelin & Alejandro, 2016; Jin et al., 2017; Ismail et al., 2020).

This study aimed to determine the genetic relationship among twelve species of the rare native *Dendrobium anosmum* Lindl. var. semialba based on the data of *matK* and *rbcL* DNA barcoding markers, which supports selective breeding and conservation of genetic resources.

2. Materials and Methods

2.1. Plant materials

Leaves of twelve *Dendrobium anosmum* Lindl. var. semialba species were collected from Son Dien village and Tam Bo village, Di Linh district, Lam Dong province, Vietnam, which were listed in the Table 1. There was a negative control sample (SD1) having a normal flower and a positive control sample (SD2) having a variant flower.

2.2. Isolation of DNA, amplification, and sequencing

Genomic DNA was extracted from 50 mg of fresh leaf explant by ISOLATE II Plant DNA kit (Bioline, UK). The isolated DNA was stored at - 20°C. The primer sets used for amplification of *matK* and *rbcL* gene were *matK*-390F (50 - eCGATCTATTCATTCAATATTTC - 30) and *matK*-1326R (50 - TCTAGCACACGAAAGTCGAAGT - 30) for *matK*, *rbcL*-aF (50 - ATGTCACCACAAACAGAGACTAAAGC - 30) and *rbcL* - ajf634R (50 - GAAACGGTCTCTCCAACG CAT - 30) for *rbcL*. A PCR reaction mixture of 50 µL comprised as follows: 2 µL of template DNA (200 ng), 1 µL of both forward and reverse primers (20 µM), 25 µL MyTaq Mix (2X) and the rest was added with deionized distilled water. PCR cycling conditions involved initial denaturation at 95°C for 1 min; followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 45°C (*matK*) or 55°C (*rbcL*) for 15 sec, and extension at 72°C for 10 sec. After 35 cycles, the PCR reaction products were held at 4°C. The PCR products were examined by electrophoresis on 1.5% agarose gel. Red gel dye was used to detect DNA lines under UV light. The band size of amplified products was determined by using a 100 bp ladder. The PCR products of *matK* and *rbcL* genes were purified. After that, the nucleotide sequences of the purified DNA were determined by Sanger sequencing ABI Genetic Analyzers 3730 XL (1st Base, Singapore).

2.3. DNA sequence data analysis

The *matK*, *rbcL* nucleotide sequences from the sequencing results of twelve *Dendrobium anosmum* Lindl. var. semialba species and the *matK*, *rbcL* nucleotide sequences of one species *Dendrobium anosmum* in the Genbank database were cut to the same size and were analyzed by Molecular Evolutionary Genetics Analysis version 7.0 (Kumar et al., 2016). The nucleotide sequences were aligned with ClustalW. The genetic distance was computed by Jukes-Cantor method. The Maximum Likelihood (ML) was used for the construction of phylogenetic trees. 1000 bootstrap replicates were executed to estimate the robustness of the ML trees (Thompson et al., 2002; Ijaz et al., 2019).

Table 1. Plant materials of species examined in this study

Sample ID	Species	Flower shape	Collection site
SD1	*Dendrobium anosmum* Lindl. (negative control)	Normal	Son Dien commune, Di Linh district, Lam Dong province, Vietnam
SD2	*Dendrobium anosmum* Lindl.var. semialba (positive control)	Variant	Son Dien commune, Di Linh district, Lam Dong province, Vietnam (received from M.M Gryshko Botanical Garden, Ukraine)
TB1	*Dendrobium anosmum* Lindl.var. semialba	Variant	Tam Bo commune, Di Linh district, Lam Dong province, Vietnam
SD3	*Dendrobium anosmum* Lindl.var. semialba	Variant	Son Dien commune, Di Linh district, Lam Dong province, Vietnam
TB2	*Dendrobium anosmum* Lindl.var. semialba	Variant	Tam Bo commune, Di Linh district, Lam Dong province, Vietnam
SD4	*Dendrobium anosmum* Lindl.var. semialba	Variant	Son Dien commune, Di Linh district, Lam Dong province, Vietnam
TB3	*Dendrobium anosmum* Lindl.var. semialba	Variant	Tam Bo commune, Di Linh district, Lam Dong province, Vietnam
TB4	*Dendrobium anosmum* Lindl.var. semialba	Variant	Tam Bo commune, Di Linh district, Lam Dong province, Vietnam
SD5	*Dendrobium anosmum* Lindl.var. semialba	Variant	Son Dien commune, Di Linh district, Lam Dong province, Vietnam
TB5	*Dendrobium anosmum* Lindl.var. semialba	Variant	Tam Bo commune, Di Linh district, Lam Dong province, Vietnam
SD6	*Dendrobium anosmum* Lindl.var. semialba	Variant	Son Dien commune, Di Linh district, Lam Dong province, Vietnam
TB6	*Dendrobium anosmum* Lindl.var. semialba	Variant	Tam Bo commune, Di Linh district, Lam Dong province, Vietnam

3. Results and Discussion

3.1. PCR amplification

The amplification success rate of the *matK* gene from twelve species of *Dendrobium anosmum* Lindl. var. semialba was 100% (Figure 1). The length of the *matK* sequences from amplification using a pair of primer *matK* - 390F and *matK* - 1326R was 936 bp. This result was similar to the study of other authors in the *matK* gene amplification (Wattoo et al., 2016). This result proved that the pair of primer *matK* - 390F and *matK* - 1326R at 45°C annealing temperatures, PCR reaction components, and thermal cycle were suitable for *matK* gene amplification in the present study.

The *rbcL* gene of all samples successfully amplified by the pair of primer *rbcL* - aF and *rbcL* - ajf634R at the annealing temperature of 55°C. The length of the *rbcL* sequences was 654 bp. It

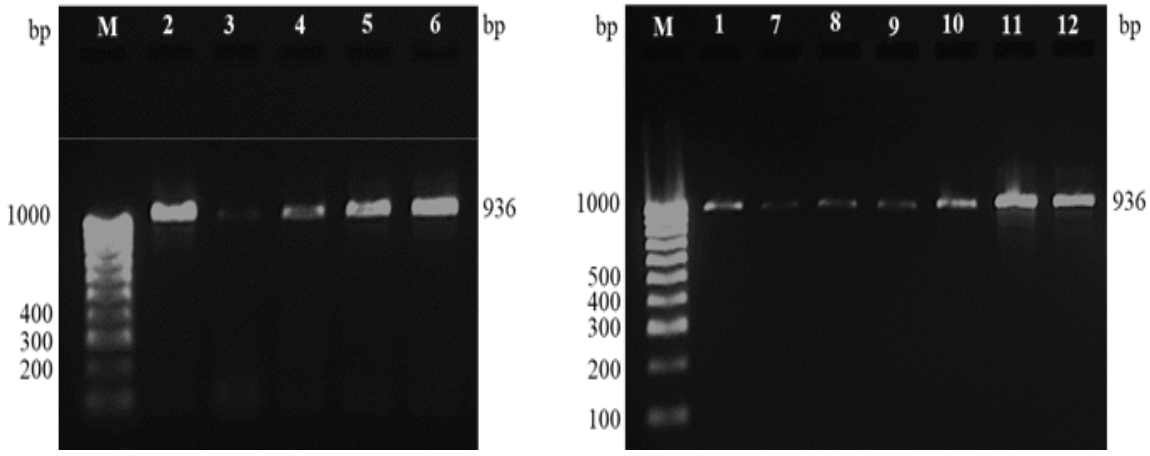

Figure 1. Electrophoresis of *matK* gene PCR products of twelve *Dendrobium anosmum* Lindl. var. semialba species. M is a 100 bp ladder, the number above each lane from 1 to 12 in turn are SD1, SD2, TB1, SD3, TB2, SD4, TB3, TB4, SD5, TB5, SD6, TB6 species).

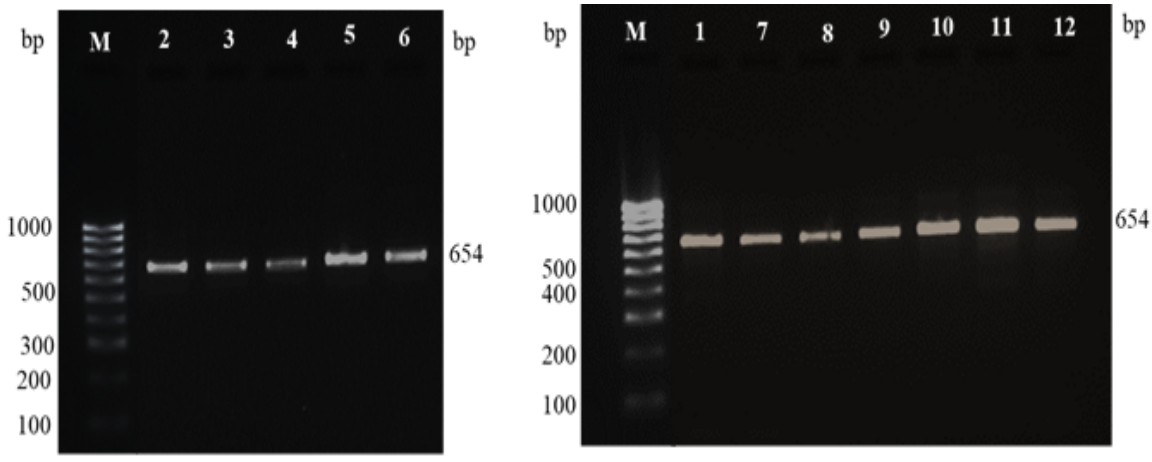

Figure 2. Electrophoresis of *rbcL* gene PCR products of twelve *Dendrobium anosmum* Lindl. var. Semialba species. M is a 100 bp ladder, the number above each lane from 1 to 12 in turn are SD1, SD2, TB1, SD3, TB2, SD4, TB3, TB4, SD5, TB5, SD6, TB6 species).

was expressed as a single line being dark bright on agarose gel (Figure 2). This proved that the PCR products had high quality for sequencing. This result was similar to the result in the study of Steven & Subramanyam (2009).

3.2. Genetic relationship analysis

The length of the *matK* sequences eliminated low-quality ends was 750 bp. The *matK* sequence was proved to be useful for *Dendrobium* species identification and reconstructing phylogeny (Asahina et al., 2010; Chattopadhyay et al., 2017). Among the chloroplast genes, *matK* is one of the most rapidly evolving genes. It has a length of about 1550 bp and encodes the enzyme maturase which is involved in the splicing of type - II introns from RNA transcripts. The rate of *matK* evolution was found suitable for resolving intergeneric as well as interspecies relationships in many angiosperms (Vijayan & Tsou, 2010). The results of aligning the *matK* sequences showed that there was only one varied nucleotide position, accounting for 0.13% *matK* sequences were analyzed (Table 2). It was a replacement of Thymine with Guanine at nucleotide positions 686 of TB1 species. The nucleotide sequences of the others were completely similar. Research by Asahina et al. (2010) also showed that individuals of the same species *D.*

Table 2. Nucleotide sequences variation in *matK*, *rbcL* gene of twelve species of *Dendrobium anosmum* Lindl. var. semialba and *Dendrobium anosmum* in the NCBI database (DA)

Species	Position[1]							
	matK	*rbcL*						
	686	281	297	369	370	422	441	469
SD1	T	C	T	T	A	G	T	A
SD2	T	C	T	T	A	G	T	A
SD3	T	C	T	T	A	G	T	A
SD4	T	C	T	T	A	G	T	A
SD5	T	C	T	T	A	G	T	A
SD6	T	C	T	T	A	G	T	A
TB1	G	A	G	T	A	T	C	C
TB2	T	C	T	T	A	G	T	A
TB3	T	C	T	T	A	G	T	A
TB4	T	C	T	T	A	G	T	A
TB5	T	A	G	C	C	T	C	C
TB6	T	C	T	T	A	G	T	A
DA	T	C	T	T	A	G	T	A

[1]Variable nucleotide positions are indicated at the top.

Figure 3. Phylogenetic tree constructed using *matK* sequences of twelve species of *Dendrobium anosmum* Lindl. var. semialba in this present study and one species of *Dendrobium anosmum* in the NCBI database (DA). Bootstrap values (%) are shown on each branch. The indicated scale represents 0.0001 nucleotide substitution per site.

moniliforme (Linn.) Swartz, D. pulchellum Roxburgh ex Lindley, *D. tosaense* Makino had no variation in the *matK* nucleotide sequence. However, there was a higher variability in *matK* sequences among *Dendrobium* species with up to 24 distinct nucleotide positions. The genetic distances between 13 species calculated using *matK* sequences were extremely low from 0 to 0.001 (Table 3). This result proved that the genetic relationship among *Dendrobium anosmum* Lindl. var. semialba species having variant flower, control species and standard species on Genbank was very close.

Data from *matK* sequence analysis were used to construct the phylogenetic tree. SD1 species having a normal flower, SD2, SD3, TB2, SD4, TB3, TB4, SD5, TB5, SD6, TB6 species having variant flower and a standard species (DA) from Genbank were gathered together with a 100% bootstrap supporting rate in the phylogenetic

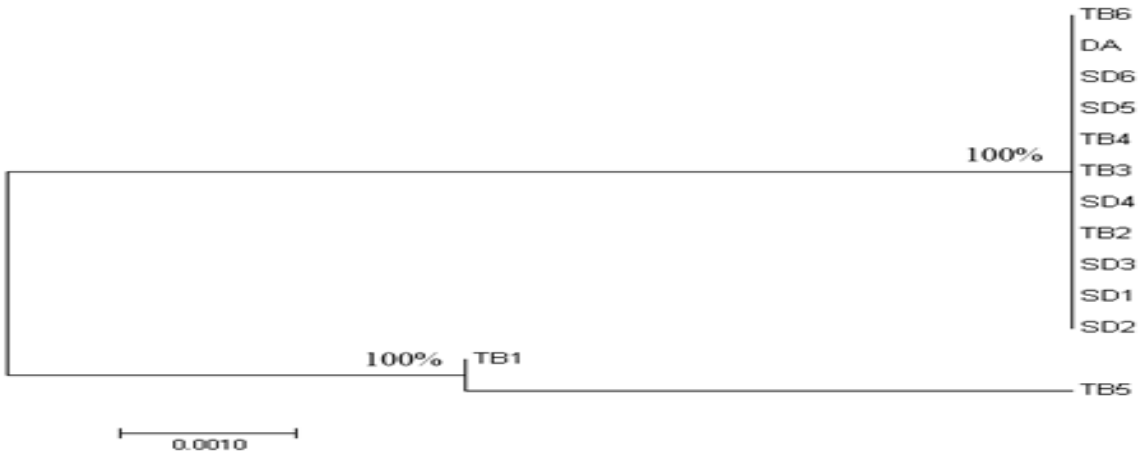

Figure 4. Phylogenetic tree constructed using *rbcL* sequences of twelve species of *Dendrobium anosmum* Lindl. var. semialba in this present study and one species of *Dendrobium anosmum* in the NCBI database (DA). Bootstrap values (%) are shown on each branch. The indicated scale represents 0.001 nucleotide substitution per site.

tree constructed using *matK* sequences, while only TB1 species was on a separate clade of the phylogenetic tree with a 100% bootstrap supporting rate (Figure 3). By contrast, another study demonstrated that 27 distinct species belonging to the same genus *Dendrobium* were grouped into many clades in the phylogenetic trees constructed based on *matK* data with a high bootstrap rate (Srikulnath et al., 2015). Therefore, the branching level of the phylogenetic tree constructed using *matK* sequences belonging to different species of genus *Dendrobium* is probably higher than that belonging to similar species of this genus.

After removing low-quality ends of amplified products of *rbcL* gene, the *rbcL* sequences were used to analyze have a length of 582 bp. *rbcL* chloroplast gene encoding ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit is the most commonly sequenced gene for phylogenetic studies of plants, especially the species of Orchidaceae family (Moudi et al, 2013b). The *rbcL* region from thirteen species *Dendrobium anosmum* Lindl. var. semialba were aligned in Table 2. The number of variable sites was seven sites in *rbcL* sequences of TB1 and TB5 and the variation rates were very low. TB1 has five single-nucleotide substitution sites, accounting for 0.86% *rbcL* sequences were analyzed. These sites were as follows: 281 (replacement of Cytosine with Adenine), 297 (replacement of Thymine with Guanine), 422 (replacement of Guanine with Thymine), 411 (replacement of Thymine with Cytosine), 469 (replacement of Adenine with Cytosine). TB5 has the most single-nucleotide substitution sites with seven sites, accounting for 1.2% *rbcL* sequences were analyzed. In addition to variable sites such as TB1, TB5 had two other variable sites were 369 (replacement of Thymine with Cytosine) and 370 (replacement of Adenine with Cytosine). Similar results between *Dendrobium* species as well as between individuals of the same species were demonstrated in the study of Asahina et al. (2010). Zhu et al. (2018) suggested that *rbcL* is unable to distinguish D. officinale from five closely related species of it. The *rbcL* gene has high conservation at the family level. Taxodiaceae (redwood) family had only minor sequence differences in their cp *rbcL* genes compared to species in the Cupressaceae (Chattopadhyay et al., 2017).

Nucleotide change plays an important role in determining genetic distance and phylogenetic origin. The *rbcL* sequences analysis showed that the genetic distances among thirteen species were low from 0 to 0.012 (Table 4). These results indicated that thirteen species in this study were closely related. From the phylogenetic trees reconstructed based on the *rbcL*, thirteen species were divided into two branches. SD1, SD2, SD3, TB2, SD4, TB3, TB4, SD5, SD6, TB6 and a species from Genbank were located in a branch; while TB1 and TB5 were located in the other branch of phylogenetic tree with a bootstrap value of 100% (Figure 4).

Table 3. Genetic distances among twelve species of *Dendrobium anosmum* Lindl. var. semialba in this present study and one species of *Dendrobium anosmum* in the NCBI database (DA) based on the *matK* sequences

Species	SD1	SD2	SD3	SD4	SD5	SD6	TB1	TB2	TB3	TB4	TB5	TB6	DA
SD1													
SD2	0.000												
SD3	0.000	0.000											
SD4	0.000	0.000	0.000										
SD5	0.000	0.000	0.000	0.000									
SD6	0.000	0.000	0.000	0.000	0.000								
TB1	0.001	0.001	0.001	0.001	0.001	0.001							
TB2	0.000	0.000	0.000	0.000	0.000	0.000	0.001						
TB3	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000					
TB4	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000				
TB5	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000			
TB6	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000		
DA	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	

Table 4. Genetic distances among twelve species of *Dendrobium anosmum* Lindl. var. semialba in this present study and one species of Dendrobium anosmum in the NCBI database (DA) based on the *rbcL* sequences

Species	SD1	SD2	SD3	SD4	SD5	SD6	TB1	TB2	TB3	TB4	TB5	TB6	DA
SD1													
SD2	0.000												
SD3	0.000	0.000											
SD4	0.000	0.000	0.000										
SD5	0.000	0.000	0.000	0.000									
SD6	0.000	0.000	0.000	0.000	0.000								
TB1	0.009	0.009	0.009	0.009	0.009	0.009							
TB2	0.000	0.000	0.000	0.000	0.000	0.000	0.009						
TB3	0.000	0.000	0.000	0.000	0.000	0.000	0.009	0.000					
TB4	0.000	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.000				
TB5	0.012	0.012	0.012	0.012	0.012	0.012	0.003	0.012	0.012	0.012			
TB6	0.000	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.012		
DA	0.000	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.012	0.000	

4. Conclusions

In summary, this study demonstrated that the *matK* and *rbcL* region sequence analyses were simple, quick, and highly reliable. Regarding species discrimination, *rbcL* gave better resolution than *matK* in identifying *Dendrobium anosmum* Lindl. var. semialba species. There was a close genetic relationship among *Dendrobium anosmum* Lindl. var. semialba species based on the low genetic distance and the minor nucleotide variation in the *matK* and *rbcL* sequences.

Conflict of interest

The authors declare no conflict of interest.

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