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A molecular phylogeny of *Taeniophyllum* THRJ inferred from DNA barcode regions

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ABSTRACT

Taeniophyllum is one of the smallest orchids in the world. This genus has around 210 species listed in the plant list. One type of this orchid is found in the Juanda Forest Park (THRJ) Bandung. This orchid is difficult to identify at the species level due to its limited morphological character and very small size. The taxonomy and conservation status of this plant are unknown; therefore, it can be used as a model for developing DNA barcodes for this genus. In this study, two DNA barcode markers (*mat*K and ITS) were used to reconstruct the phylogenetic relationship of *Taeniophyllum* THRJ. By using *mat*K sequences, *Taeniophyllum* THRJ was grouped with *Taeniophyllum glandulosum* and *T. aphyllum*. However, using the ITS sequence, *Taeniophyllum* THRJ was positioned together with *Microtatorchis* sp., *T. smithii*, and *T. complanatum*. The results showed that the *mat*K gene can be used for DNA barcoding of *Taeniophyllum* orchids. The use of the ITS sequence for the *Taeniophyllum* group still cannot be confirmed yet. Based on *mat*K sequences and possibly ITS sequences, it can be concluded that *Taeniophyllum* THRJ is not *T. glandulosum* but is another species of *Taeniophyllum*.

INTRODUCTION

As a high source of germplasm, Indonesia has a variety of orchids, starting from the largest orchid (*Grammatophyllum speciosum*) to the smallest orchid (*Taeniophyllum* sp.) in the world. Orchid conservation needs to be done considering its active compounds that are potential to be developed as medicines from natural ingredients. The active compounds found in orchids include alkaloids, terpenoids [1-3], and phenols [4]. Approximately 494 soil and epiphytic orchid species, 49 of them were used as traditional medicines in Africa to treat cough, symptoms of diarrhea, treat pain, and worms [5].

Taeniophyllum is one of the genera of the Orchidaceae family (orchids) and consists of about 221 species. This orchid can be found in Indonesia, Malaysia, the Philippines, and several places in China, Japan, Africa, and Australia. This smallest orchid population decreases due to the limited number of host plants. *Taeniophyllum* can only grow on meranti (*Shorea* sp.) trees. Meranti trees are woody plants that are often used as materials for building houses and furniture. Also, the host plant is increasingly diminished due to habitat loss caused by several factors including the conversion of land into residential and agricultural areas. This in turn will have an impact on the survival of this orchid.

To overcome the constraints of lack of taxonomists and plant characters, DNA-based identification has been developed. One such method is DNA barcoding. This method has been used for species delineation and

analyze species' genetic diversity [6]. This DNA-based method is increasingly used because it is easier to do, faster, and provides more accurate results than morphological identification [7]. Therefore, this method has been used in the fields of taxonomy, phylogenetics, and biodiversity analysis.

The taxonomists in the world have begun to turn to DNA barcoding technology for the identification of orchids. Several studies of orchid barcoding DNA have been carried out in previous studies [8 – 11]. This technology, besides being used to accurately identify species, can also be used for the reconstruction of phylogenetic trees and to know the kinship between species. The genes used as DNA barcodes for plants are *ribulose bisphosphate carboxylase large chain* (*rbc*L), *maturase* K (*mat*K), *intergenic spacers* (IGS) such as *nuclear ribosomal internal transcribed spacer* 1 (nrITS 1) and 2 (nrITS 2), as well as *chloroplast* IGS which lies between the transfer of RNA for leucine and phenylalanine (cp *trn*L-*trn*F IGS) [12]. In DNA barcoding methodology, two standard genes have been approved and recommended by the Barcode of Life (CBoL) Consortium. Both of these genes are *rbc*L and *mat*K [13]. In various studies, the *mat*K gene was used more frequently than the *rbc*L gene because the *mat*K gene was even more difficult to apply but provided a higher resolution compared to the *rbc*L gene [14]. The *mat*K gene is considered to be more accurate in identifying because it can differentiate up to the species level while the *rbc*L gene can only distinguish up to the genus level [15]. However, the best DNA barcodes for *Dendrobium* orchids are loci 18S-ITS1-5.8S-ITS2-28S [12]. Given the importance of accuracy in the identification process, then the purpose of this study is to evaluate several DNA barcodes to be used in identifying the smallest orchid in the world, *Taeniophyllum* THRJ, and its taxonomic implications.

MATERIALS AND METHODS

Sample preparation

The plant was obtained from Juanda Forest Park Bandung, West Java, Indonesia (coordinate 6°51′24″S 107°37′57″E). A sample of 50 mg of orchid roots was crushed in an Eppendorf tube using a mini pestle. DNA extraction was performed using the Plant Genomic DNA Mini Kit (Geneaid Biotech, New Taipei, Taiwan) according to the manual procedure provided. Lysis buffer was added to the tube then incubated for 10 minutes at 65°C to optimize the cell wall lysis process. The lysed cells were then separated by centrifugation for 1 minute at a speed of 5000 rpm, then added with a buffer, and followed by centrifugation for 2 minutes at 10000 rpm. The supernatant was then filtered through the filter column. The total DNA obtained from the centrifugation process was washed from the remains of protein and salt. Furthermore, DNA was eluted for 2-5 minutes and centrifuged for 30 seconds at 10000 rpm.

Amplification of DNA barcode regions

The amplification process was done using MyTaq HS Red Mix (Bioline, Meridian Bioscience, London, UK), which consists of 20 µl, 1.5 µl (10uM) of each primer, 2 µl DNA template, 15 µl MiliQ water. The primer pairs used in this research for amplification and sequencing for each DNA barcode can be seen in Table 1. Amplification condition was as follow: Denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and polymerization at 72°C for 50 seconds. Final polymerization was done at 72°C for 1 minute [15].

Table 1. Primer pairs used for DNA amplification and sequencing.

DNA barcode	Primer pairs	Reference
***mat*K**	matK-1RKim: ACCCAGTCCATCTGGAAATCTTGGTTC matK-3FKim: CGTACAGTACTTTTGTGTTTACGAG	[16]
***rbc*L**	rbcLa-F: ATGTCACCACAAACAGAGACTAAAGC rbcLa-R: GTAAAATCAAGTCCACCRCG	[17][18]
ITS (universal)	ITS92: AAGGTTTCCGTAGGTGAAC ITS75: TATGCTTAAACTCAGCGGG (ITS1, 5.8S-coding region dan ITS2)	[19]
***trn*H–*psb*A**	trnHf_05: CGCGCATGGTGGATTCACAATCC psbA3_f: GTTATGCATGAACGTAATGCTC	[20] [21]

Phylogenetic analysis

The phylogenetic analysis was performed using the stepwise procedure developed previously [22]. The chromatograms were processed using Geneious 10.1.3 [23]. The primer sequences were removed by trimming approximately 50 nucleotides at the beginning of DNA sequences. Errors of the reading of the nucleotides were corrected accordingly. Consensus sequences were generated using a pairwise alignment of forward and reverse sequences. All similar sequences obtained from Genebank were aligned using multiple sequence alignment with hierarchical clustering [24], which is available online at http://multalin.toulouse.inra.fr/multalin/, and trimmed accordingly to obtain the core sequence. The history of evolution is concluded using the Maximum Likelihood method based on the Tamura-Nei model [25]. Tree construction was carried out using 1000 x bootstrap by applying Neighbor-Joining and BioNJ algorithms on a pairwise distance matrix estimated using the Maximum Composite Likelihood (MCL) approach. Evolutionary analyses were conducted in MEGA7 [26].

RESULTS

Morphology of *Taeniophyllum* THRJ

The orchid (Figure 1) has green terete roots, approximately 1 mm in diameters. The flowers were yellowish green with a 2-3 mm long tube of sepals.

Figure 1. Root and flowers of *Taeniophyllum* THRJ.

Amplification of DNA barcode regions

The regions of DNA barcode which were successfully amplified were only rbcL, matK, and ITS (Figure 2), while the region trnH–psbA failed to be amplified using primer pairs stated in Table 1.

Kimura's 2-parameter model was used to estimate the genetic distance (Table 2) because it can show base substitution per site between sequences of *mat*K genes. The genetic distance of *Taeniophyllum* THRJ was 0.039 (3.9%) with *T. glandulosum* and 0.044 (4.4%) with *T. aphyllum*. This implies this plant is more closely related to *T. glandulosum* than *T. aphyllum*. However, with a genetic distance of 0.033 (3.3%), *T. aphyllum* is more related to *T. glandulosum* than *Taeniophyllum* THRJ. This can assume that *Taeniophyllum* THRJ is neither *T. glandulosum* nor *T. aphyllum*.

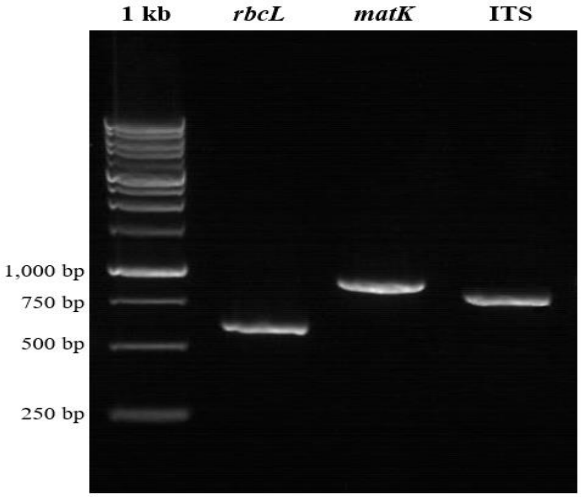

Figure 2. The DNA barcode regions of *rbc*L and *mat*K genes, and ITS which were successfully amplified.

Sequencing results showed that using the *mat*K gene, the *Taeniophyllum* THRJ has an identity of 96.1% with *Taeniophyllum glandulosum* voucher Z.J.Liu 5458 (KJ733612.1) [27], and 95.6% with *Taeniophyllum aphyllum* 5458 (AB217766.1) [28]. The results of phylogenetic tree reconstruction (Figure 3). the *Taeniophyllum* THRJ is closely related to *T. glandulosum* and *T. aphyllum* (clade 2). Both clusters 1 and 2 are from the Aeridinae subtribe.

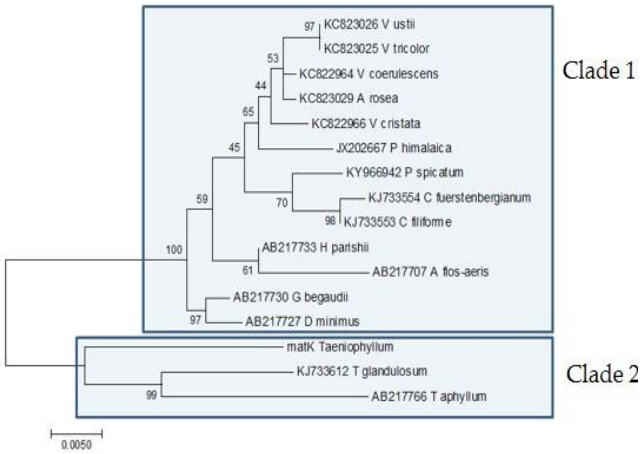

Figure 3. Phylogenetic tree reconstruction of *Taeniophyllum* THRJ of *mat*K gene (assigned as *mat*K *Taeniphyllum*) using maximum likelihood method. Grouping by similarity to sequences is indicated. Proportions of bootstrap are indicated near the nodes.

Table 2. The genetic distance of *mat*K gene of *Taeniophyllum* THRJ with its allied taxa using Kimura's 2-parameter method.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	matK_*Taeniophyllum* THRJ																
2	KJ733612 *T. glandulosum*	0.039															
3	AB217766_*T._aphyllum*	0.044	0.033														
4	AB217733_*H._parishii*	0.047	0.049	0.056													
5	AB217730_*G._begaudii*	0.048	0.049	0.053	0.011												
6	AB217727_*D._minimus*	0.048	0.051	0.055	0.012	0.006											
7	AB217707_*A._flos-aeris*	0.051	0.060	0.064	0.011	0.022	0.023										
8	KY966942_*P._spicatum*	0.052	0.057	0.064	0.015	0.019	0.021	0.016									
9	KJ733554_*C._fuerstenbergianum*	0.052	0.055	0.061	0.016	0.018	0.019	0.019	0.012								
10	KJ733553_*C._filiforme*	0.052	0.055	0.061	0.013	0.017	0.018	0.017	0.010	0.002							
11	KC823029_*A._rosea*	0.052	0.053	0.060	0.010	0.012	0.013	0.017	0.011	0.013	0.011						
12	KC823026_*V._ustii*	0.052	0.053	0.060	0.013	0.014	0.016	0.021	0.016	0.016	0.013	0.005					
13	KC823025_*V._tricolor*	0.052	0.053	0.060	0.013	0.014	0.016	0.021	0.016	0.016	0.013	0.005	0.000				
14	KC822966_*V._cristata*	0.052	0.053	0.060	0.013	0.013	0.014	0.019	0.013	0.014	0.013	0.006	0.008	0.008			
15	KC822964_*V._coerulescens*	0.052	0.056	0.062	0.012	0.014	0.016	0.019	0.013	0.016	0.013	0.002	0.005	0.005	0.006		
16	JX202667_*P._himalaica*	0.052	0.056	0.062	0.016	0.018	0.019	0.023	0.018	0.021	0.018	0.011	0.013	0.013	0.012	0.011	

Table 3. The genetic distance of ITS of *Taeniophyllum* THRJ with its allied taxa using p-distance method.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	ITS92-7 *Taeniophyllum* THRJ														
2	DQ091723 *Microtatorchis* sp.	0.731													
3	DQ091726 *T. smithii*	0.729	0.636												
4	AB217590 *T._aphyllum*	0.724	0.715	0.743											
5	AB217578 *P. pallidum*	0.745	0.721	0.764	0.651										
6	DQ091724 *T. complanatum*	0.715	0.652	0.606	0.720	0.703									
7	AB217580 *S. pusillum*	0.719	0.693	0.745	0.671	0.638	0.724								
8	AF537008 *P. maculata*	0.722	0.674	0.715	0.651	0.607	0.706	0.628							
9	AY912232 *P. kunstleri*	0.738	0.707	0.716	0.338	0.637	0.713	0.665	0.626						
10	AB217582 *S. chrysanthus*	0.719	0.703	0.737	0.680	0.649	0.748	0.290	0.641	0.677					
11	AB217563 *M. griffithii*	0.719	0.707	0.733	0.685	0.649	0.750	0.307	0.636	0.677	0.006				
12	AY273752_*P._gigantea*	0.719	0.721	0.749	0.725	0.726	0.717	0.711	0.714	0.730	0.733	0.733			
13	KY966412 *A. parviflora*	0.703	0.530	0.693	0.685	0.684	0.736	0.625	0.679	0.686	0.617	0.619	0.734		
14	KJ733455 *T. glandulosum*	0.694	0.734	0.765	0.618	0.628	0.723	0.642	0.663	0.664	0.652	0.652	0.711	0.689	

Table 4. The summary of genetic distance using *mat*K gene and ITS sequences.

		*mat*K Kimura's 2-parameter			ITS p-distance		
		1	2	3	1	2	3
1	*Taeniophyllum* THRJ						
2	*T. glandulosum*	0.039			0.694		
3	*T. aphyllum*	0.044	0.033		0.724	0.618	

Using *rbc*L gene for identification, *Taeniophyllum* THRJ has 98% identity with *Drymoanthus adversus* voucher CHR:596828 (KT007199.1), *Gastrochilus japonicus* voucher PDBK2015-1266 (KX871236.1), *Gastrochilus calceolaris* voucher CPG25086 (KX527436.1), *Gastrochilus obliquus* voucher CPG25085 (KX527435.1), and *Plectorrhiza tridentata* FN870901.1.

Identification of the specimen using ITS sequence showed that *Taeniophyllum* THRJ has an identity of 90% with *Microtatorchis* sp. (DQ091723.1), 89% with *Taeniophyllum smithii* (DQ091726.1), *Taeniophyllum aphyllum* (AB217590.1), *Pteroceras pallidum* (AB217578.1), and *Taeniophyllum complanatum* (DQ091724.1). Genetic distance calculation using p-distance for ITS can be seen in Table 3. *Taeniophyllum* THRJ has a genetic distance of 0.745 with *P. pallidum* AB217578, 0.738 with *P. kunstleri* AY912232, 0.731 with *Microtatorchis* sp. DQ091723, 0.729 with *Taeniophyllum smithii*, 0.724 with *T. aphyllum*, 0.715 with *T. complanatum*, and 0.694 with *T. glandulosum*. Figure 4 shows phylogenetic tree reconstruction of *Taeniophyllum* THRJ using ITS sequences. In this tree, *Taeniophyllum* THRJ is clustered together in clade 2 with *Microtatorchis, P. gigantea, T. smithii,* and *T. complanatum*. On the other hand, *T. glandulosum* and *T. aphyllum* are clustered in clade 1.

The summary of genetic distance using *mat*K gene (Kimura's 2-parameter) and ITS (p-distance) is shown in Table 4. Both results agree that *Taeniophyllum* THRJ is closer to T. *glandulosum* than *T. aphyllum*, while *T. glandulosum* is closer to *T. aphyllum* than *Thaeniophyllum* THRJ.

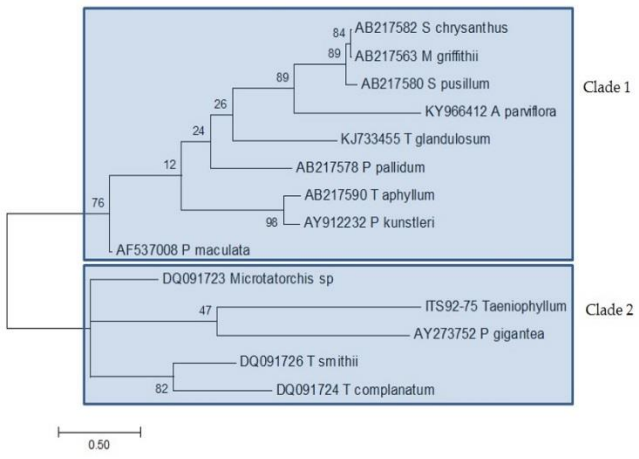

Figure 4. Phylogenetic tree reconstruction of *Taeniophyllum* THRJ ITS sequence using maximum likelihood. Grouping by similarity to sequences is indicated. Proportions of bootstrap are indicated near the nodes.

DISCUSSION

The two genes, *mat*K and *rbc*L, are coding genes recommended as DNA barcodes for terrestrial plants [29]. Of them, the *mat*K gene has the most varied regions of the angiosperms plant group. By using *mat*K, it was found that *Taeniophyllum* THRJ is closely related to *T. glandulosum* and *T. aphyllum*. However, estimation of genetic distance using Kimura's 2-parameter indicates that *Taeniophyllum* THRJ is not *T. glandulosum* nor *T. aphyllum*. This fact is supported by the previous statement that a minimum intraspecific distance of orchid *Dendrobium* is 0 and maximum is 1.14%, and a minimum interspecific distance is 0 and maximum is 10.1% [9]. For orchid *Paphiopedilum*, the maximum intraspecific and intraspecific distances were 1.5% and 4.5%, respectively [11]. Because of the genetic distance between *Taeniophyllum* THRJ with *T. glandulosum* (3.9%) or with *T. aphyllum* (4.4%) greater than 1.5%, it is certain that *Taeniophyllum* THRJ obtained from Tahura Juanda Bandung is not *T. glandulosum* nor *T. aphyllum.* An interesting fact is, however, that *Taeniophyllum aphyllum* Makino is a synonym of *Taeniophyllum glandulosum* Blume [30]. Using a molecular approach, the genus *Microtatorchis* (Schltr.) was combined with *Taeniophyllum*, therefor currently genus *Microtatorchis* has been renamed as genus *Taeniophyllum* [31].

Taxa kinship will get closer if it has a small genetic distance value [15]. For example, based on the *mat*K gene, the intraspecific distance is 0.14% for *Astragalus* (Fabaceae) [32], 0.26% for *Acacia* (Fabaceae) [33], 0.5% for the family Myristicaceae [34], and 0.16% for Rosaceae [35].

The use of the rbcL sequence did not get the proper identification results. We assume that there no information on the *rbc*L sequence of *Taeniophyllum* in GenBank. We also assume that the *rbc*L gene cannot be used for *Taeniophyllum* THRJ species identification. A previous study also provided the view that the *rbc*L sequence cannot be considered as an identification tool for closely related species of *Dendrobium* [36].

The maximum intraspecific distance of ITS for *Dendrobium* using p-distance was 0.82 [9], while the genetic distance of *Taeniophyllum* THRJ and *Taeniophyllum glandulosum* is 0.694, and *T. aphyllum* is 0.724. This also reinforces the notion that *Taeniophyllum* THRJ is not *T. glandulosum* nor *T. aphyllum.*

However, due to the large genetic distance using *mat*K and ITS sequences, it can be summarized that *Taeniophyllum* THRJ is not *T. glandulosum* nor *T. aphyllum*. To be assigned a new name, the results must be accompanied by the scrutinization of morphological character. Nevertheless, this finding implies that revisitation of *Taeniophyllum* taxonomy and reclassification has to be done.

CONCLUSIONS

It can be concluded that the *mat*K gene can be used as a core DNA barcode in the *Taeniophyllum* group, possibly in combination with the ITS sequence. This finding concludes also that *Taeniophyllum* THRJ found at Juanda forest park in Bandung is not *T. glandulosum* nor *T. aphyllum*.

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AUTHOR CONTRIBUTIONS

TET and TBE conceived and design the analysis; LL collected the samples and verified the analytical method; JJP and BJK contributed data analysis; and TET, SM, and TBE wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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