DNA barcoding of *Thrixspermum longipilosum* based on Internal Transcribed Spacer 2 (ITS2) region

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**Abstract.** *Thrixspermum* is an orchid that has the characteristics of a very short flowering period. This is one of the obstacles in the conventional identification process that uses morphology, both vegetative and generative. For this reason, a molecular approach is needed as an alternative identification tool for the *Thrixspermum* orchid. For molecular identification, CBOL recommends three markers, namely *matK*, *rbcL*, and *ITS*. In this study, only *ITS2* was used since *ITS* have been reported for its ability to discriminate plant sample up to species levels. The genomic DNA of *T. longipilosum* was isolated using the CTAB method with minor modification, then used as a template in PCR amplification. The amplicon resulted from the PCR reaction (± 450 bp in size) was purified and further determined using DNA sequencer. The results showed that *T. longipilosum* has a high homology level with its close relatives (per id 94.07%-96.91%). From alignment analysis, the *ITS2* sequence of *T. longipilosum* showing differences nucleotides located in sequences 3 (A→G), 55 (G/A→T), and 64 (A→G).

Meanwhile, phylogenetic analysis showed that *T. longipilosum* formed one cluster with *T. linusi*. This shows that *ITS2* are highly recommended as a molecular marker to determine the barcode of the *T. longipilosum*.

**Keywords:** DNA marker, molecular identification, orchid

1. Introduction

Indonesia is a country that has high biodiversity in which 25% of flowering plants are in this country. One of the flowering plants family is Orchidaceae with a total species up to 25,000 species belong to 870 genera [1] and 5,000 species were located in Indonesia [2]. Nowadays, deforestation will threaten the existence of orchids that life epiphytically in the forest tree, and one of these is *Thrixspermum* orchid.

*Thrixspermum* is an orchid that has the characteristics of a very short flowering period, and one of the species is *Thrixspermum longipilosum*. This orchid lives epiphytes by attaching to tree trunks [3] and spread over the western Sumatera. *Thrixspermum* orchid species have synonym and high
similarity characters between species [4], resulted in the difficulty for the identification process through morphological observation. Therefore, an alternative identification based on molecular characters (DNA barcoding) is required.

DNA barcoding is a molecular method to identify species quickly and accurately based on DNA sequences. This method contributes to various ecological and conservation studies [5]. Some advantages of DNA barcoding among others only require a few or small specimens for identification, being able to document the diversity of taxonomic groups that have never been identified, and being able to reveal new variations or new variations in species that were previously classified as a single species [6].

The Consortium for the Barcode of Life (CBOL) recommends three markers for plant DNA barcoding, namely matK, rbcL, and ITS (ITS1 and ITS2), but in this study only ITS2 was used. ITS2 is not only useful for DNA barcoding but also as a standard molecular marker for orchid taxonomies [7]. Extensive research has focused on chloroplast genes and introns [8]. ITS2 was chosen as a barcode candidate because ITS2 is a potential molecular marker and is widely used for phylogenetic reconstruction at the genus and species level [9]. Therefore, this research was conducted which aims to identify short sequences as potential barcodes on Thrixspermum longipilosum based on ITS2 region.

2. Materials and Method

2.1 Genomic DNA Isolation
Genomic DNA of T. longipilosum was extracted using Cetyl Trimethyl Ammonium Bromide (CTAB) method followed by the treatment with NEXprep DNA Kit (Korea). Leaf sample (0.1 g) was crushed in CTAB buffer using sterile mortar and pestle. The homogenate was then added 5 μL of RNAse (10 mg/ml) and incubate at 65 °C for 15 minutes. The next steps were performed following the manufacture’s instruction of NEXprep DNA Kit (Korea).

2.2 PCR Analysis & Electrophoresis
The PCR reaction consists of 1.5 μL DNA template, 10 μl 2 × Go Taq® Green PCR Master Mix (Promega, USA), 1 μL of both forward and reverse primers (10 pmol) (DR2F, 5’-GGCTCTCGCATC GATGAAGA-3’ and ITS_26SE, 5’-TAGAATTCCCGGTCTCGGCATGC-3’), and deionized distilled water until total volume of 20 μL. PCR programs used were as are follows denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min 15 sec, then final extension at 72°C for 5 min and hold at 16°C. The PCR were performed for 35 cycles. The amplified PCR product then loaded on agarose electrophoresis gel at 100 Volt for 30 min and visualized on UV transilluminator.

2.3 PCR Product Purification & Sequencing
PCR product purification was carried out to obtain pure DNA and free from contaminants. The amplified PCR product was purified using Jena Bioscience Purification Kit and the nucleotide sequences (Adenine, Thymine, Guanine, and Cytosine) were determined by employing the sequencing service provided by Macrogen, Korea.

2.4 Sequence Analysis
Some bioinformatics software used in sequence analysis were BLAST NCBI to confirm ITS2 DNA sequence and homology level of T. longipilosum, Clustal X v1.8.7 to align nucleotide, and MEGA7 for phylogenetic construction.

3. Result and Discussion
The genomic DNA isolated from *T. longipilosum* was used as a template for amplification of ITS2 using Polymerase Chain Reaction (PCR) machine in 35 cycles. DNA amplification based on PCR using ITS2 primer set showed a single and clear DNA band at the size about 450 bp (Figure 1). This result shows that the DNA was perfectly amplified by the ITS2 primer set with appropriate PCR condition. This is in consistent with the statement that ITS2 is easy to be amplified [10].

The sequence of amplified ITS2 was determined using DNA sequencer, and results then analyzed using some bioinformatics software. The homology of the amplified sequence was determined using Basic Local Alignment Tool (BLAST) program. This analysis aims to find out the homology level of the organisms based on ITS2 sequence [11]. The results showed that the ITS2 sequence was able to distinguish the sample up to the species level (Table 1).

![PCR product amplification of ITS2 region of Thrixspermum longipilosum](image)

**Figure 1.** PCR product amplification of ITS2 region of *Thrixspermum longipilosum*, M = 1kb DNA Marker, Tl = *Thrixspermum longipilosum*

Based on the results of NCBI BLAST analysis, it is known that *T. longipilosum* shows a high level of homology (94.07%-96.91%) with its close relatives (Table 1). *T. longipilosum* has the highest homology level (96.91%) with *T. linusii* (KX679333) from Malaysia. In addition, there are 9 different base sequences in these two species, located at nucleotide number 3 between Guanine (G) and Adenine (A), nucleotide number 55 between Thymine (T) and Adenine (A), nucleotide number 64 between Adenine (A) and Cytosine (C), at nucleotide 114 between Cytosine (C) and Thymine (T), at nucleotide number 120 between Thymine (T) and Guanine (G), at nucleotide 130 between Cytosine (C) and Guanine (G), at nucleotide 181 between Cytosine (C) and Adenine (A), at nucleotide 258 between Adenine (A) and Guanine (G), and at nucleotide 290 between Adenine (A) and Thymine (T) (Figure 2). *T. longipilosum* also has high homology with other types of *Thrixspermum*, namely *T. triangulare* (96.04%), *T. japonicum* and *T. caudatum* (94.93%), *T. centipeda* (94.71%), *T. tortum* (94.67%), and *T. merguense* (94.07%). The zero (0) E-value showed the alignment of all sequences was significant value [12]. Alignment result shows that *T. longipilosum* has 3 bases that distinguished *T. longipilosum* from other species, located at nucleotide sequence number 55 with Thymine (T), 64 with Adenine (A), and 321 with Thymine (T). ITS2 sequence shows variety and was able to discriminate to the species.

**Table 1.** BLAST result analysis of *T. longipilosum* with other species in NCBI

| Name | Accession Number | % Ident | E-value | Source |
|------|------------------|---------|---------|--------|
|      |                  |         |         |        |
Figure 2. Alignment of ITS2 sequence from *T. longipilosum* with closest relatives in NCBI

Phylogenetic tree construction is the most important thing in the study of the evolution of an organism [13]. The phylogenetic tree shows that *T. longipilosum* and *T. linusii* form one cluster. It can be known that both have a close genetic relationship with bootstrap value is 92% (Figure 3). The phylogenetic tree above was constructed with a bootstrap value of 1000. The bootstrap value in the phylogenetic tree aims to measure how many replications in the phylogenetic tree are constructed [14]. Bootstrap value on a clade of 70% or more means the clade was acceptable [15]. The results of the phylogenetic reconstruction show compatibility between the BLAST results and the phylogenetic tree.
Figure 3. Neighbor-joining tree based on analysis of the ITS2 region in *T. longipilosum*

4. Conclusion

The ITS2 sequences of *T. longipilosum* have unique characteristic and was able to discriminate up to the species level. The genetic relationship shows that the *T. longipilosum* originating from the same ancestor with *T. linusii*. Based on these results, ITS2 sequences are highly recommended as a molecular marker to determine the barcode of the *T. longipilosum*.

5. References

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