Direct PCR for DNA barcoding of *Bulbophyllum lobbii* Lindl. based on *rbcL* sequence

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Abstract. DNA barcoding is a molecular technique frequently used to identify or confirm a species, which involves the steps of isolation, amplification via PCR, and sequencing analysis. However, the use of lysate derived from samples after soaked and heated in TE buffer is lacking reported for this application. This study aims to provide an alternative method for PCR using lysate as template for species identification of *Bulbophyllum lobbii* using *rbcL* primers. The results show that the lysate (after heating and briefly spun) is worthy of use as a template in PCR amplification, able to produce a thick single band with appropriate amplicon size (±600 bp). Further sequencing analysis confirms that the resulting sequence is highly readable with a clear chromatogram. BLAST analysis shows high identity (100%) with *Bulbophyllum lobbii* (MT518983) from USA. In sum, direct PCR using lysate provides an alternative approach for rapid DNA barcoding of plant samples with promising results.

1 Introduction

DNA barcoding is an important tool for molecular identification of species using short DNA sequences or fragments that represent the characteristics of a particular organism [1]. In accordance with the recommendation of The Consortium for the Barcode of Life (CBOL), two types of markers can be used, namely *matK* and *rbcL*, with one supporting marker, namely ITS [2]. The use of the three barcodes has been used to identify various plants, including several orchids such as Phalaenopsis [3], Dendrobium [4], Thrixspermum [5], dan Bulbophyllum [6].

The molecular identification process consists of several stages including: isolation of DNA from samples, amplification using PCR, sequencing, and analysis of obtained sequences data using several bioinformatics software [5], [6], [7]. For DNA isolation, researchers mainly use the CTAB method [8]. The advantage using this method is the isolated DNA has a large amount or quantity, but has a drawback in terms of time (the process takes hours). Although the problem of the length of time that arises in the CTAB method can be overcome by using a kit from the company, another problem that arises is the high price of the kit. A breakthrough method is needed to overcome these two obstacles, and we proposed the direct PCR method.

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Several direct PCR methods have been developed for certain purposes. For example, Jung *et al.* utilized direct PCR for studying low biomass organisms, such as lichens, cyanobacteria, and cryptogams [9]. While Jeon *et al.* applied direct PCR to amplify the ITS region of the fungi *Fusarium fujikuroi* [10]. In addition, direct PCR was employed for amplification of housekeeping genes in several genetically engineered plants with reproducible results [11]. Although there are some examples of direct PCR applications as mentioned above, still there is no report about the utilization for molecular identification purposes, especially for orchids DNA barcoding. The aim of this study was to test the application of direct PCR for the purpose of molecular identification of *Bulbophyllum lobbii* orchid using the *rbcL* marker.

2 Materials and Methods

This study used *Bulbophyllum lobbii* Lindl. collected from Gumitir mount and grown in the green house as described previously [6]. The research was conducted from January to October 2024 in the Biotechnology sub-laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Jember.

The leaves were taken using a perforator (diameter 5 mm) and placed in a microtube contained 500 µl of Tris-EDTA (TE) buffer. The samples were vortex for 10 seconds, then incubated at 55°C in a thermo-shaker for 15 minutes. Afterward, the samples were centrifuged at 10,000 rpm at 25°C for 3 minutes. Finally, 250 µl of the liquid (supernatant) was transferred to a new tube and applied as the PCR template. For each PCR reaction, the mixture consists of 10 µl green master mix, 6 µl nuclease-free water, 2 µl template solution, 1 µl forward primer *rbcL*_F (5'-ATGTCACCACAAACAGAGACTAAAGC-3'), and 1 µl reverse primer *rbcL*_R (5'-GTAAAATCAAGTCCACCRCG-3') [5]. The amplification process includes three stages: initial denaturation at 95°C for 5 minutes; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute and 20 seconds; and a final extension at 72°C for 5 minutes [4].

Analysis of the target DNA amplification (amplicon) results was carried out using electrophoresis by loading on a 1.25% agarose gel for about 30 minutes with a voltage of 100 Volts. The electrophoresis results were then visualized by placing the gel on a UV-transilluminator to confirm the existence of DNA bands. Subsequently, the PCR product was subjected to sequencing by sending to Biotechnology company for DNA sequencing service, and the data obtained were then analysed using several bioinformatics software such as BioEdit [12], BLAST [13], ClustalX [14], and MEGA11 [15].

3 Results and Discussion

One of the important stages that must be passed for molecular identification is DNA amplification using PCR which requires genome DNA that must be isolated either using the CTAB method, kit, or other methods. In this study, we propose a faster and more economical strategy through the direct PCR method using lysate obtained from a short soaking and heating process. The results show that the soaking process in TE buffer followed by heating at temperature of 55ºC can help providing materials that function as a template for reaction in the PCR machine. With the composition of the mixture and standard PCR conditions as described in the materials and methods, the PCR product shows single thick band and in accordance with the predicted size, which is around 600 bp without any non-specific band observed (Figure 1). In addition, the PCR results can be compared with the PCR results obtained by the standard method using CTAB and the method using the kit that we have previously published [3], [5], [6]. The results utilizing the direct PCR method are very

reliable since in DNA barcoding work, single- and thick bands would affect the next stage i.e. sequencing as one of the important stages in molecular identification process.

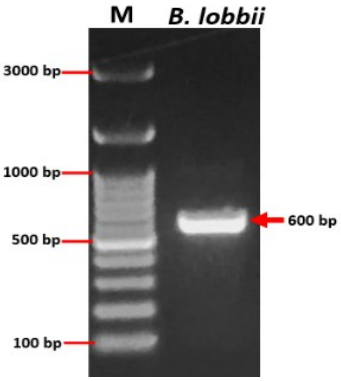

Fig. 1. PCR product amplified from *Bulbophyllum lobbii* through Direct PCR method using *rbcL* as primer set. M: DNA marker.

To ensure that the PCR product obtained is *rbcL* fragment, the sequencing was performed by sending the sample to sequencing service company. The sequencing results were then analysed using Bioedit, BLAST, and ClustalX softwares. Bioedit is used to ensure that the peaks formed in chromatogram are errorless according to the nucleotide bases appeared in the sequence data. BLAST is used to compare the sample sequence with existing sequences of *Bulbophyllum lobbii* (*B. lobbii*) and other plants deposited in the NCBI Genbank, while ClustalX is applied to align the sample sequence with the selected sequences retrieved from Genbank to discover whether any differences in nucleotide bases. As shown in Table 1, the BLAST results showed that the sample sequence had 100% identity with *B. lobbii* and *B. dearei* with accession numbers MT518983 and MT518970, respectively. In addition, the sample sequence had 99.81% identity with *B. lilacinum*, *B. patens*, and *Lepanthes rekoi* with accession numbers KF421861, MT519000, and OP711527, respectively. These results support the fact that with direct PCR method, the resulting sequence is comparable with the sequence obtained from the existing standard procedure for DNA barcoding steps in general. This results also support previous studies applying direct PCR for other purposes with reliable results [9], [10], [11].

Table 1. BLAST analysis of *rbcL* sequence from *Bulbophyllum lobbii* sample with *rbcL* sequences deposited in NCBI GenBank database.

Scientific Name	Max Score	Total Score	Query Cover (%)	E. Value	Per. Ident.	Acc. Len. (bp)	Accession/ Origin
Bulbophyllum lobbii	955	955	100	0.0	100	553	MT518983/ USA
Bulbophyllum dearei	955	955	100	0.0	100	549	MT518970/ USA
Bulbophyllum lilacinum	950	950	100	0.0	99.81	631	KF421861/ Bangladesh
Bulbophyllum patens	950	950	100	0.0	99.81	549	MT519000/ USA
Lepanthes rekoi	950	950	100	0.0	99.81	587	OP711527/ USA

The *rbcL* sequence of *B. lobbii* was then analysed for alignment with five sequences deposited in Genbank as shown in the Table 1. The results showed that the *rbcL* sequence of the Bulbophyllum orchids had one nucleotide difference with the *Lepanthes rekoi* orchid at

the 380th nucleotide base, namely cytosine (C) in Bulbophyllum and Adenine (A) in Lepanthes. Meanwhile, at the 454th, there is a difference nucleotide between *B. lobbii*, *B. dearei*, with *B. lilacinum* and *B. patens*, namely in the Thymine (T) and Cytosine (C) bases (Figure 2).

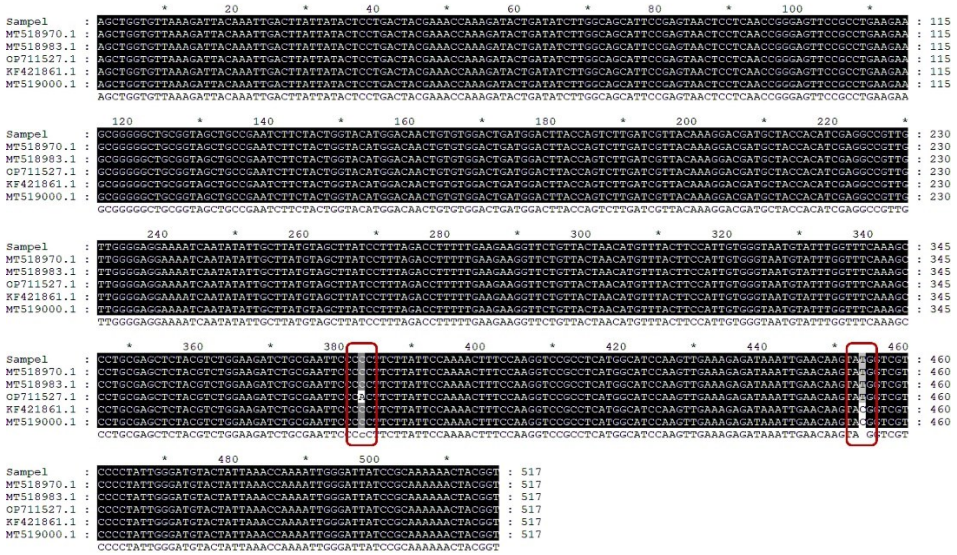

Fig. 2. Alignment of *rbcL* sequence of *Bulbophyllum lobbii* with *rbcL* sequences from related orchids available in NCBI database. The differences in nucleotide sequences between species are highlighted in red boxes.

To further support the readability of sequence alignment data, a phylogenetic tree construction was performed. As expected, the results are more visible in the phylogenetic tree illustration (Figure 3) which shows that the Bulbophyllum orchid (sample) is located in one cluster with *B. lobbii* and *B. dearie* (GenBank), while *B. lilacinum* is in one cluster with *B. patens*. In contrast, *Lepanthes rekoi* is not located in a cluster with any Bulbophyllum orchids (Figure 3).

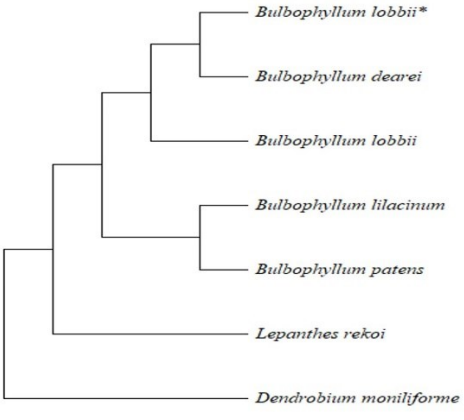

Fig. 3. Phylogenetic tree of *Bulbophyllum lobbii* sampel (*) with other related orchids based on *rbcL* sequences. *Dendrobium moniliforme rbcL* sequence was selected as outgroup.

4 Conclusion

The Direct PCR method developed in this research shown potential for DNA barcoding purpose without requiring DNA isolation procedures. Two advantages of this method are time-efficient and affordable cost, and applicable for huge number of samples. Therefore, this method can be an alternative in DNA barcoding for other orchids species with reliable results.

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