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Phylogenetic Analysis of *Phanerochaete chrysosporium ITB* Isolate Using Internal Transcribed Spacer (ITS) Sequence

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Abstract: The aim of this research is to conduct phylogenetic analyzes of *Phanerochaete chrysosporium ITB* isolate using DNA sequences of the internal transcribed spacer (ITS) region. The rDNA ITS sequence was developed by using universal ITS5/ITS4 primer pairs. The results showed that the DNA sequence of the ITS region of 700 bp length of *Phanerochaete chrysosporium ITB* has the highest similarity of 99.6% with *PhanerochaetechrysosporiumBKM-F-1767, RP78, PV1, KCTC 6728, SF-4, ATCC MYA-476, FCL208, FCL236,* and Gold-9-419-4. *Phanerochaete chrysosporium ITB* isolate suspected as new strain of *Phanerochaete chrysosporium.*
Keyword: *Phanerochaete chrysosporium*, ITS, phylogenetic.

Introduction

Phanerochaete chrysosporium is a white wood rot fungus belonging to Basidiomycetes[1]. *P. chrysosporium* has been known to have the ability to degrade lignin and a variety of aromatic pollutants. This ability is caused by the possession of lignin peroxidase (LiP), manganese peroxidase (MnP) and H_2O_2-producing oxidase enzyme. Those three enzymes are the major component of lignolytic extracellular enzymes produced by the organism under conditions of limited nitrogen, carbon and sulfur[2]. However, it was also reported that *P. chrysosporium* produces very small amounts of laccasein a particular medium[3].

*Phanerochaete chrysosporium*is utilized in various fields of industry and biotechnology due to its ability to produce lignolytic enzymes. *P. chrysosporium* is used to generate natural aromatic flavorings in the food industry. It's used as a biocatalys in bleaching process and decolorize of kraft pulp mill effluent of paper and pulp industry. Its use is to increase the quality of the pulp. *P. chrysosporium* has been utilized in a variety of bioremediation processes because of its ability to degrade various types of dyes, i.e. azo, reactive and heterocyclic dyes, 1.1.1-trichloro-2.2-bis-(4-chlorophenyl) ethane (DDT), 2,4,6-trinitrotoluene (TNT) and polycyclic aromatic hydrocarbons (PAH's), and also pesticides and other xenobiotics. Currently, LiP and MnP from *P. chrysosporium* are being developed as biosensors and biocatalysts in several chemical processes[4].

P. chrysosporium ITB isolate has been widely used as subjects in many study. This isolate is one of collection of Microbiology Laboratory InstitutTeknologi Bandung allegedly owned since 1999.Previous research shows that the isolate is unique because the ability to produce MnP lowest than LiP even in specific media for production MnP[5] whereas *P. chrysosporium* BKMF-1767 (ATCC 24725) which has been widely studied can generate MnP with high activity[6,7,8]. Molecular identification through phylogenetic analysis

is needed to determine the validity and relationship of the isolate with other isolates of *P. chrysosporium.*Beside, some species among *Phanerochaetes* generally have similar morphological characters thatmisidentified strains when deposited in culture collections or original strains were contaminated during subsequent transfers can occur[9].

Phylogenetic analysis performed by using internal transcribed spacer (ITS) sequence. ITS is part of ribosomal RNA-coding genes in eukaryotic genomes. ITS region became more potential DNA barcode for fungi than mitochondria cytochrome c oxidase subunit 1, LSU RNA polimerase II, second LSU RNA polimerase II, minichromosom maintenance protein, LSU and SSU nuclear ribosomal[10]. One unit of genes encoding ribosomal RNA sequence consists of a small area subunit (SSU) 18S, ITS 1, 5.8 S, ITS2 and large subunit (LSU) 28S respectivly. SSU, 5.8 S and LSU is a coding region, whereas the ITS1 and ITS2 are noncoding areas[11]. The regional of ITS 1 and ITS 2 genes flanking the 5.8 S are generally have a size of 300-900 bp[12].

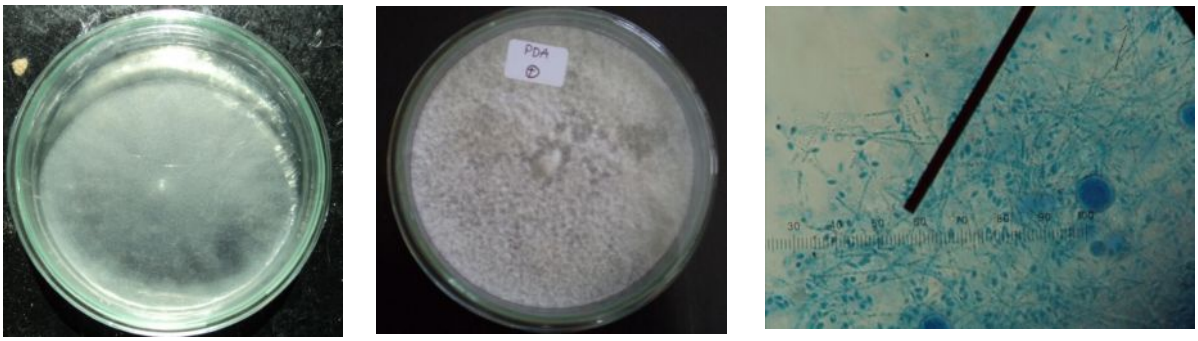
Experimental

Phanerochaete chrysosporium isolate identified in this research was a collection of Microbiology Laboratory InstitutTeknologiBandung. The isolate was grown on Potato Dextrose Broth (PDB) medium in 25 mL Erlenmeyer flask at 30^{o}C. The inoculum was agitated for three days using a shaker with the speed of 120 rpm. Genome DNA was extracted from fungal mycelium according to the Doyle & Doyle method[13] with some modifications i.e: cell lysis was done by grinding the mycelium mass using poly etilena glycol (PEG) 600, adding lysis buffer and β-mercaptoetanol, incubating at 65^{o}C for 1 hour, then the mixture was added directly with phenol-chloroform-isoamilalcohol (PCI) solution.

DNA amplification was performed using ITS5 (5'-GGAAGTAAAAG TCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers. The PCR mix composition and the amplification conditions refer to Lestari[14]. PCR results were visualized by electrophoresis on 2% agarose gel and observed using UV transiluminator. Purification of PCR product and sequencing were performed at Macrogen, South Korea.

A total of 19 ingroup reference isolates (*P.chrysosporium*) and 4 outgroup reference isolates (*Phanerochaetesordida*) from the GeneBank (http://www.ncbi.nlm.nih.gov) were presented in FASTA format. Phylogenetic analysis was done according Lestari[14] that alignment of DNA sequences was done using ClustalW program. Phylogeny tree was constructed using Phylogeny in MEGA 5.03 program with 1000 bootstrap, inversion using Maximum Likelihood methods with Tamura-Neialogaritm and evolutionary distance were analyzed according to Tamura-Neialgoritm.

Result and Discussion



A **B** **C**

Figure 1.Characteristic of Morphology of *Phanerochetechrysosporium* ITB isolate: colony in the "Difco" PDA medium on the 5th day (A), on the 14th day (B) and microscopicmorphology on the 9th day (C).

*Phanerochaetechrysosporium*ITB isolate has the following morphology (Figure 1): *First*, the colony of *Phanerochaetechrysosporium*ITB isolateis slow growth rate (± 5 days), yellowish white of pigmentation the upper colonies; white pigmented the bottom colonies; irregular, flat, uneven edges, continuous and diffuse, upper rough colony structures such as cotton and sticky. *Second*, the microscopy characteristic shows it has

septat, unbranched and smooth-walled hypa; globular, thick-walled and single sporangium; unbranched sporagiofor and observed the presence of clamydiosfor. The results of these observations in accordance with the morphological characters of *Phanerochaete chrysosporium*[15].

Ampliconof *Phanerochaetechrysosporium* ITB which amplified with ITS4 and ITS5 primers resulted a single band of approximately 900 bp size in gel electrophoresis (Figure 2), whereas 707 bp based on the sequen DNA of the amplicon. These results are almost similar with the size of ITSsequen of reference isolate from NCBI.

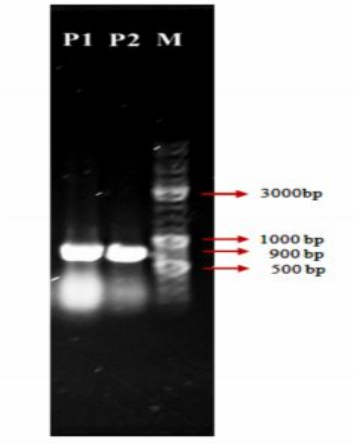


Figure 2. DNA Amplicons with ITS4/ITS5 primer, P1 and P2 is *Phanerochaetechrysosporium*ITB, while M is a marker (GeneRuler™ DNA Ladder Mix 100

Based on phylogenetic analysis (Figure 3) showed that the value of similarity with the reference isolate (ingroup) is 99,6-95,10 %, while the outgroup is 94-95 % (Tabel 1). It also produced phylogenetic trees as shown in Figure 3. Interpretation of the results based on the phylogenetic species concept was conducted that *Phanerochaete chrysosporium ITB* was confirm a *Phanerochaete chrysosporium* but suspected as a new strain because do not have 100% similarity with any reference isolate. The phylogenetic spesies concept stated that between isolates are the same strain if the similarity value of 100 %, the same species if the minimum similarity value of 99 % and the same genus if the similarity value between 89 - 99 %[14,16,17]. This is confirmed by the analysis of polymorphic between *Phanerochaete chrysosporium ITB(PC)* and the nearest reference isolate (*PhanerochaetechrysosporiumBKM-F-1767*). Both strain were not have parsimony information but just two single tone that is on the order of 398 and 412 bases. The guanine base on 398 PCs into thyiminepada on *PC BKM-F-1767* and adenine at 412 bases into guanine. This result also explain about the diference mangan peroxidase profil of this isolate.

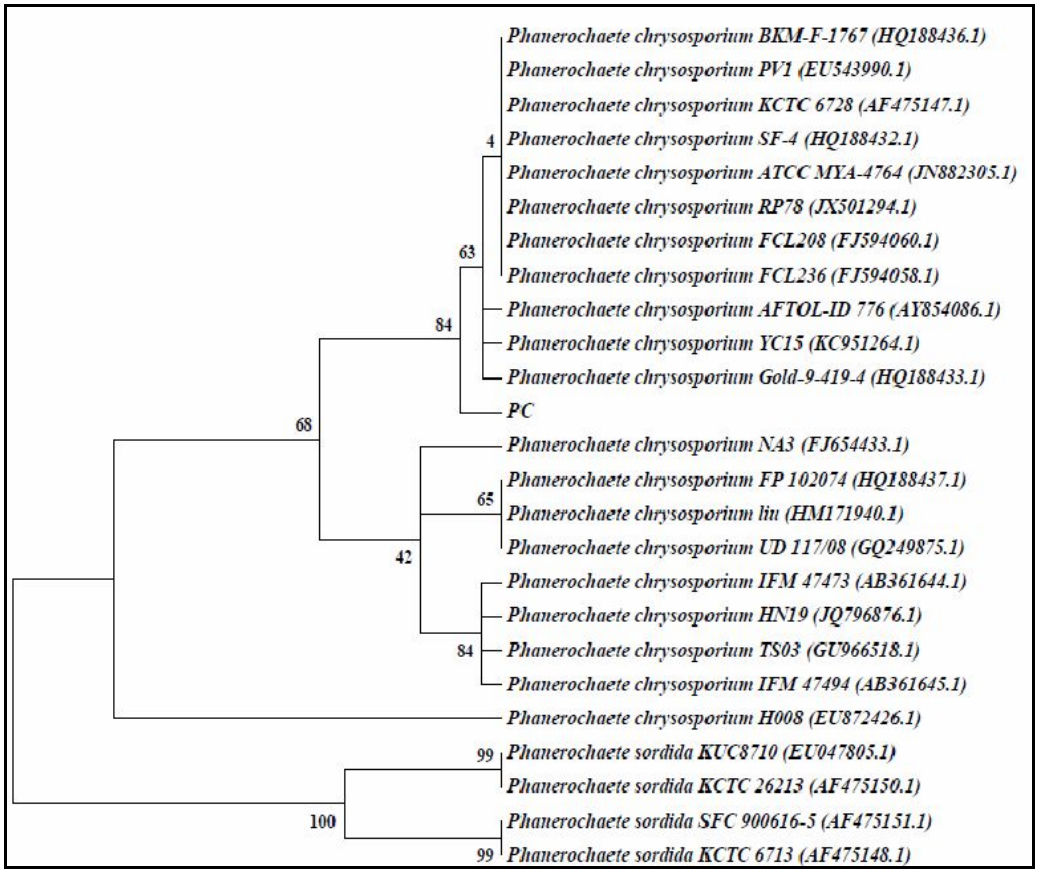


Figure 3. Phylogeny Tree of *PhanerochaetechrysosporiumITB* and Reference Isolates based on Maximum Likelihood Algorith with Tamura-Nei Methods Analysis

Table 1. The Similarity Value Between*Phanerochaetechrysosporium ITB* Compare with The Reference Isolates

The Reference Isolates	Similarity Value
PhanerochaetechrysosporiumBKM-F-1767 *PhanerochaetechrysosporiumRP78* *Phanerochaetechrysosporium PV1* *Phanerochaetechrysosporium KCTC 6728* *Phanerochaetechrysosporium SF-4* *Phanerochaetechrysosporium ATCC MYA-4767* *Phanerochaetechrysosporium FCL208* *Phanerochaetechrysosporium FCL236* *Phanerochaetechrysosporium* Gold-9-419-4	99,60 %
Phanerochaetechrysosporium AFTOL-ID 776 *Phanerochaetechrysosporium YC 15*	99,40 %
Phanerochaetechrysosporiumliu	98,40 %
Phanerochaetechrysosporium UD 117 *Phanerochaetechrysosporium FP102074*	98,39 %
Phanerochaetechrysosporium NA3	98,19 %
Phanerochaetechrysosporium IFM 47494	97,79 %
Phanerochaetechrysosporium HN19 *Phanerochaetechrysosporium TS03*	97,80 %
Phanerochaetechrysosporium H008	95,10 %
Phanerochaetesordida KUC8710 *Phanerochaetesordida KCTC 26213*	95,09 %
Phanerochaetesordida SFC 900616-5 *Phanerochaetesordida KCTC 6713*	94,65 %

The results of the analysis carried out also showed that not all its regions sequences of reference isolates meet phylogenetic species concept. There are eleven reference isolates of nineteen isolates suggested to be used as reference isolates for identification phylogenetic of *Phanerochaetechrysosporium*. Presumable, this is

because the length of the ITS sequences from each isolate was not exactly the same and there are a few sequences that do not read no bases.

Conclusion

The results of phylogenetic analysis using ITS sequence showed that *Phanerochaete chrysosporium ITB* has the highest similarity of 99,6% with *PhanerochaetechrysosporiumBKM-F-1767, RP78, PV1, KCTC 6728, SF-4,ATCC MYA-476, FCL208, FCL236,* andGold-9-419-4. It is suspected as a new strain of *Phanerochaete chrysosporium*

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