Identification of Fungi Based on ITS from Humus Soil at UNILA, Indonesia.

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ABSTRACT

Fungal isolate HEU1 isolated from humus soil at UNILA, Indonesia was identified based on Internal Transcribed Spacer (ITS). Fungal isolate HEU1 has brown blackish spore and white mycelia. DNA was isolated from mycelia and the ITS region was amplified by Polymerase Chain Reaction (PCR) with ITS4 and ITS5 primers. The PCR product was purified using PCR Purification Kit, and nitrogen base sequence analyzed with automated DNA sequencer. The homology of sequences analyzed with Basic Local Alignment Search Tool nucleotide (BLASTn). The BLASTn result showed that the fungal isolate HEU1 has 100% homology with Aspergillus aculeatus strain M9. ClustalW was used for sequence-alignment. Phylogenetic analysis was constructed using Neighbour joining method with Kimura distance. The phylogenetic tree obtained showed that fungal isolate HEU1 is Aspergillus aculeatus.

KEYWORDS: Aspergillus, Internal transcribed spacer, phylogenetic tree, DNA (Deoxyribo Nucleic Acid) sequence.

INTRODUCTION

Identification of fungi has an aim to discover nature diversity of fungi species. Identification proceeds with comparing the unknown isolate with existing taxa to establish the identity [1]. Fungi identification can be done in two ways, conventional and molecular. Conventional identification of fungi can be done based on morphology, physiology, and biochemical characteristic. Morphology and physiology character from two species closely related fungi, often identical, which can lead miss-identification. Therefore, the fungi identification is required molecular way. Molecular identification method is using Deoxyribo Nucleic Acid (DNA). Regions of genes encoding ribosomal RNA (rRNA) or ribosomal DNA (rDNA), is a common target (DNA). Regions of genes encoding ribosomal RNA (rRNA) or ribosomal DNA (rDNA), is a common target

ITS regions are non-coding regions that have higher mutation rates than coding regions, thus generally have a higher sequence variation than coding regions [4]. One potential advantage of this method is that ITS primers designed according to the highly conserved rRNA gene sequences, could be highly applicable in a broad range of organisms including plants, fungi and animals [5]. According to Sugita et al., the same species have regional ITS sequence homology 99-100%, while the homology lower than 99% showed a distinct species [6].

Previous research by Husniati et al., showed that isolate HEU1 can produce galactomannan, but in those research isolate still known as A. niger [7]. In this research, we clarify and underline our statement about the identification of the isolate. Samson et al., stated that species which belong to Aspergillus section Nigri were A. aculeatus, A. brasiliensis, A. carbonarius, A. costaricensis, A. ellipticus, A. japonicus, A. foetidus, A. heteromorphus, A. homomorphus, A. lactofoetatus, A. niger, A. piperis, A. sclerotiorigen, A. tubingensis, and A. vadensis [8]. A. niger section Nigri have been extensively used for various biotechnological purpose, including production of enzyme and metabolites to be used in food industry. A. niger products hold the GRAS (Generally Recognized As Safe). Due to their biotechnological importance, the identification of such strain needs to be unambiguous. This research aimed to identify fungal isolate HEU1, isolated from Unila humus soil, based on Internal Transcribed Spacer (ITS).

MATERIALS AND METHODS

Materials
The material used in this study were humus soil at University of Lampung (UNILA) as a sample source culture, Potato Dextrose Agar (Difco), peptone Water (Pronadisa), distilled water, lactophenol (Merck).

Methods
Isolation
Five grams of humus soil samples taken from the UNILA student village’s (Kampung Baru) dissolved in 45 mL of distilled water and measured the pH. Samples were then diluted to 10-12 dilution factors. After that,
pipetted and poured in PDA media, this work was performed in duplicates. Samples were then incubated for 3-7 days at 25 °C.

**Morphology Identification**

Observations were done from day-1 until day-7. Parameters analyzed included: changes arising on colony color, colony surface conditions (flat, mounting, powders, granules, grain, velvet or cotton), presence or absence of radial lines, the availability of lines or concentric circles, presence or absence of exudates drops, the bottom of the colony and the presence or absence of odor.

Microscopic observation was using lactophenol. Observations performed with 10 x 1000 zoom. Parameters analyzed included: the availability conidiophores, conidia and vesicles.

**Molecular Identification**

Molecular identification of fungi carried out based on genetic analysis as partially on ribosomal DNA sub unit of fungi that includes internal transcribed spacer (ITS4 and ITS5). PCR amplification using Primer ITS4 at 5’- TCC TCC GCT TAT TGA TAT GC - 3’ and Primer ITS 5 at 5’- GGA AGT AAA AGT CGT AAC AAGG-3’[5].

PCR product purification was done by PCR Purification Kit and followed by cycle sequencing. Results returned purified cycle sequencing with Ethanol purification method. Analysis of the reading order of nitrogen bases using automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems).

Sequence data then trimming and assembling, with the BioEdit program, and further conversed in a FASTA format. The results then run for BLAST for looking homology, in data base center of the DNA at the site http://www.ncbi.nlm.nih.gov [9].

**Phylogenetic Tree**

Establishment of a phylogenetic tree based on Hall [10]. Analysis of the others sequences accessed from GenBank, which are *Aspergillus aculeatus* strain M9 (GenBank JQ670921.1), *A. japonicus* strain CBS 61 178 (GenBank: AY585562.1), *A. carbonarius* strain SRRC (GenBank: AY373844.1), *A. niger* strain ASRA2 (GenBank: JQ675290.1), *A. ochraceus* isolates 41 (GenBank: HQ905486.1), and *Penicillium chrysogenum* M30 strain (GenBank: JQ422624.1) as outgroup.


**RESULTS AND DISCUSSION**

**Morphology Identification**

Macroscopic observations of fungal isolates HEU1 on the 7th day incubation at temperature 25° C on PDA showed same characteristic with genus *Aspergillus* [14], the spore colors were brown to black, with colonies mounting surface were white colored cotton and colonies diameter were 80 mm (Picture 1).

![Picture 1](image1.png)

**Picture 1.** Macroscopic view of fungi isolate HEU1 (days 7) on PDA.

Microscopic observations showed that *Aspergillus* isolates had small conidia colored dark brown, uniseriate, spherical vesicle shapes and forms fiallid like tubes (Picture 2). *Aspergillus* species that uniseriate were *A. aculeatus*, *A. aculeatinus*, *A. japonicus* and *A. uvarum* [14,15].
Molecular Identification

Sequences result from automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer):

>Aspergillus aculeatus, 458 bp
TCCTTCGGGGCCCAACCTCCACCCGTTCCTACCTGGTTCTCCGCGGCGCCGGCCGCCCTTCCGGGC
CGGCCGGCCGGCCTGCCGGGCCTGGCCCGCCGGCCGGGAGACCCCAATGGAAACTGCTTGAGCTCAT
CGATGAAGAAGCGAGGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCAGTC
TTGAAACGCACATTGCGCCTTGCTACGGCGACGTCACTTTCCTCCCTC
AGCAGCCCCGTGTTGTTGCGGCGCAGCCCCCGGGGGCGGGCCGGGCGGTCTAGAGTAAAGAACGCGG
CCCCTGGTATTCCCGGGGGCATGCCTGTCCGAGCGTCATTTCTCCCCTC

Identification was done by homology search on the ITS region sequence, using BLASTn program on DNA database GenBank. The BLASTn showed that the sequence of fungus isolates HEU1 have 100% homology with *A. aculeatus* strain M9 (Appendix 1). According to Sugita et al., isolates with ITS region sequences ≥ 99% with the closest species was the same species [6]. Therefore, it can be concluded that the fungal isolates HEU1 was identified as *Aspergillus aculeatus*, grouped in *Aspergillus* section Nigri [8].

Phylogenetic Analysis Of Fungal Isolate HEU1

FASTA data from the base sequence was converted into Mase with ClustalW and Seaview4 program, then Phylogenetic Tree-Neighbour joining (NJ) of isolate HEU1 constructed with phylo_win program, using Kimura distance. Schematic phylogeny with bootstrap was done 1000 times (Picture 3).

![Phylogenetic tree](image3.png)
Phylogenetic tree showed that *A. aculeatus* (isolate HEU1) have closely related with *A. aculeatus* strains M9 and the *A. japonicas* strain CBS, and *Penicillium chrysogenum* strain M30 as an outgroup. Al-Musallam (1980) stated that *A. aculeatus* Iizuka as varieties of *A. japonicas*, but Raper and Fennell (1965) stated that *A. aculeatus* and *A. japonicas* were two different taxa [in 15]. Those statement also supported by Pafenicová et al., that examined 9 *Aspergillus japonicus* isolates and 10 *Aspergillus aculeatus* isolates by using molecular and biochemical markers, including DNA sequences of the ITS1-5.8S rRNA gene-ITS2 region, restriction fragment length polymorphisms (RFLP), and secondary-metabolite profiles. The DNA sequence of the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene could not be used to distinguish between *A. japonicus* and *A. aculeatus* but did show that these two taxa are more closely related to each other than to other species of black aspergilli [17].

From the phylogenetic tree above can be seen that *A. aculeatus* was different taxa to *A. japonicus*, showed from its bootstrap number is 100.

**Conclusion**

The observation of macroscopic, microscopic, and molecular identification confirmed that fungal isolate HEU1 isolated from UNILA humus soil is *Aspergillus aculeatus*.

**REFERENCES**


Appendix 1. Data alignment sequence isolate HEU1 with A. aculeatus strain M9 on BLASTn analysis.