Molecular Identification of Decay Fungi in Xylem of Yellow Meranti (*Shorea gibbosa*) Canker

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Abstract

By molecular technique, identification of decay fungi isolated from decayed xylem of the tree canker of yellow meranti (*Shorea gibbosa*) have been carried out. In this molecular identification, the ITS (Internal Transcribed Spacers) region of rDNA of fungal isolates were amplified and sequenced. The DNA sequence for each fungus was aligned with that of known species in the ITS region of rDNA database for fungi. By phylogenetic analysis based on the sequences of the ITS region of rDNA, a basidiomycete fungi isolated from decayed xylem of yellow meranti was identified as *Phlebia brevispora*. The presence of ascomycete fungi on the decayed xylem samples was also detected and identified as *Aspergillus nomius*, *Phlogicylindrium* sp., *Hypocrea rufa*, *Hypocrea lixii*, *Talaromyces* sp., and *Trichoderma virens*.

Key words: decay fungi, ITS region of rDNA, phylogenetic analysis.

Introduction

Molecular methods are increasingly being used to identify wood-decay fungi (Claudia *et al*. 2000; Cortesi *et al*. 2000; Blanchette *et al*. 2004; Diehl *et al*. 2004; Kim *et al*. 2005). These methods provide a rapid and accurate way of characterizing and identifying organisms because they rely on objective information (molecules) derived from the target organism rather than the substantial subjectivity of classical methods (Diehl *et al*. 2004; Schmidt 2006). This approach has also provided a wealth of information about phylogenetic relationships (Hibbett and Donoghue 2001) which is useful for taxonomic research and classification of organisms.

The most efficient method of deriving a useful level of information is to sequence a well-characterized region of DNA, then compare this sequence with that of known fungi stored in public and private databases. The ribosomal DNA internal transcribed spacers (rDNA ITS) region is a genomic region widely used in fungal systematics and identification. Its advantages lie in the highly conserved nature of the ribosomal DNA genes, which means that primers can be designed to amplify DNA from a broad range of species, and the high degree of variation in the non-coding region. As the spacers region does not code for a functional gene, there is little, if any, constraint on mutations in this region.

Information on the identity of decay fungi that degrade xylem of living trees can be used in many ways. Accurate identification of decay fungi on decayed tissue will be especially useful in investigations of xylem decay to establish precise methods for controlling decay and to solve the problem of decay in forest trees.

Shorea gibbosa, which grows in the lowlands of the Southeast Asian tropical rainforest, is known as member of the yellow meranti group of timbers. The timbers have been used in the manufacture for plywood, fiberboard, particleboard, moulding, and other building materials due to their high physical properties. Because such forests are managed for timber production, decay that develops on the tree stems reduces grade quality or timber yield, and is therefore a serious economic problem.

In previous study, it was reported that the patterns of xylem decay of *S. gibbosa* were similar to those of white-rot fungi (Erwin *et al.* 2007). Therefore, we intended to identify decay fungi isolated from the decayed xylem of *S. gibbosa* by using sequences of the ITS region of ribosomal DNA (rDNA ITS) to ascertain and confirm the presence of white-rot decay fungi inhabiting the decayed xylem.

Materials and Methods

Xylem Sample Collection

Xylem samples were collected from 35-year-old *S. gibbosa* cankerous tree (diameter at breast height (DBH) approximately 40 cm) growing in a natural dipterocarp stand at the Bukit Soeharto Educational Forest of Mulawarman University, East Kalimantan, Indonesia. The transverse view of the stem canker *S. gibbosa* show what appear to be decayed in the exposed xylem at the canker margin. The decay appeared to extend from outside to inside and also tangentially (Figure 1a).

One disk 5 cm thick was cut from the cankered stem of *S. gibbosa*. From the cankered stem disk, xylem samples (approximately 15 x 15 x 15 mm) were taken from each of the four sites of decayed xylem and sound ones.

Fungi Isolation

The medium used for isolating xylem-decaying fungi was 2% malt extract agar (pH 5.5) (20 g of malt extract and 15 g of Difco agar in 1000 ml distilled water) with 100 ppm of chloramphenicol (antibiotic) designated as MEA, and 2% MEA with 100 ppm of chloramphenicol and 100 ppm of benomyl (a fungicide with broad-spectrum activity on ascomycetes) designated as BMEA. All media were sterilized by autoclaving at 0.103 MPa (121°C) for 20 min.

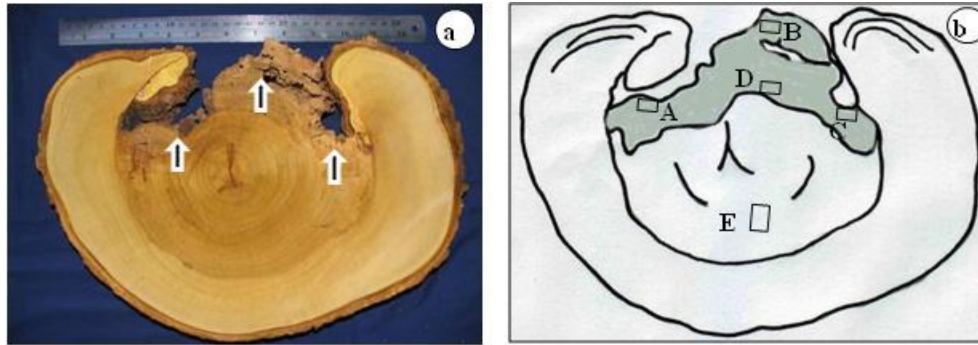

Figure 1. (a) Cross cut surface of *S. gibbosa* with exposed xylem showing decay at the canker margin (arrows). (b) Sketch of describing the zoning of the cut surface and the sites of sample collection *S. gibbosa*. Note decayed zone is shaded gray: decayed xylem in the canker margin (A~C), decayed xylem adjacent to the region of sound xylem (D), and sound xylem opposite the region of decayed xylem (E).

Three small pieces of xylem (dimension: approximately 5 x 5 x 5 mm) were cut from decayed and sound sites on the disk (Figure 1b). All xylem samples were aseptically transferred to culture media in petri dishes and then incubated at room temperature (24°C) for 14 days. A small piece of agar containing fungal mycelium was transferred separately from the margin of the colony growing from each of the xylem samples onto MEA to purify the cultures. To obtain selective growth of basidiomycete fungi was transferred to BMEA medium. Finally, 30 isolates were isolated. Each fungal isolate was categorized into morphotype based on its colony appearance, growth pattern, and spore morphology, if available. One isolate arbitrarily selected from each morphotype was later transferred to 5 ml of 2% malt extract liquid medium (20 g of malt extract in 1000 ml of distillated water) designated as ME in test tubes and incubated at room temperature for 3~5 days. Prior to transferring the fungal isolate, the ME was sterilized as previously described. The young mycelium was used for DNA extraction.

DNA Extraction and Purification from Fungal Mycelia

Fungal mycelium (corresponding to ca. 2 mg after drying) was transferred directly from liquid culture using a sterile inoculation wand into a microtube for DNA extraction. DNA extraction and PCR conditions generally followed the method described by Matsuda and Hijii (1999), which were modified from that of Gardes and Bruns (1993).

PCR Amplification

The ITS region and 5.8S gene of rDNA of fungal isolates were amplified using PCR with total reaction volume of 25 μl (template DNA, ca. 0.5 ng/μl; each dNTPs, 200 μM; Taq DNA polymerase (New England Biolabs), 0.5 U; each primer, 0.5 μM, Tris-HCl, 1 mM; KCl, 5mM). The PCR mixture was prepared according to the manufacture's instruction. A primer set, ITS 1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), designed by White *et al*. (1990), was used; however, when the PCR was unsuccessful, the forward primer ITS1F was replaced by an alternative primer, ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') also designed by White *et al*. (1990). The PCR condition followed the method of Gardes and Bruns (1993).

DNA Sequencing and Analysis

The purified PCR products were sequenced according to the method as described by White *et al*. (1990), for the ITS1, ITS2, and 5.8S regions of nuclear ribosomal DNA, using the primers ITS1F, ITS5, and ITS4. DNA sequences were determined using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, CA, USA). The DNA sequence determined for each fungus was aligned together with that of known species in the GenBank database [National Centre for Biotechnology Information (NCBI) US National Institute of Health Bethesda (http:/www.ncbi.nlm.nih.gov/)]. A computer software "GENETYX-MAC Ver.11.2" was used for the alignment. Identification at the genus level was based on identities above 95% following the method of Landeweert *et al*. (2003). For basidiomycetes, further analysis was conducted using Clustal W version 1.83 (1994) via the online analysis service of the DDBJ (http://clustalw.ddbj.nig.ac.jp/top-e.html). A default set of the parameters was used for the alignment and phylogenetic analysis.

Phylogenetic relationships were inferred using the "Bootstrap N-J" (neighbor-joining) tree program (Saitou and Nei 1987) in Clustal W based on Kimura-2-parameter distance (Kimura 1980). To evaluate the strength of support for the branches of the N-J trees, 1000 replications of bootstrap analysis were performed. The tree was displayed using TreeView PPC 1.6.6 (Page 2001).

Results and Discussion

Isolation from decayed xylem samples cultured on BMEA and MEA media yielded 8 fungal isolates for *S. gibbosa*. Based on the results of DNA sequence analyses shown Table 1, only one fungal isolate (YM3ac) was matched to basidiomycete.

Table 1. Information on DNA sequences of the fungal isolates from the decayed xylem of *S. gibbosa* examined in this study and the most similar species registered in GenBank database.

Code name[a]	Accession number[b]	Closest match[c] (accession number)	Homology Value[d] (bps)
YM3ac	AB297801	*Phlebia brevispora* (AB084614)	98% (456)
YM3	AB297795	*Aspergillus nomius* (DQ467992)	100% (437)
YM4	AB297796	*Phlogicylindrium eucalypti* (DQ923534)	87% (563)
YM8	AB297797	*Hypocrea rufa* (AJ230677)	98% (589)
YM10	AB297798	*Phlogicylindrium eucalypti* (DQ923534)	87% (563)
YM14	AB297799	*Trichoderma virens* (AF099007)	100% (596)
YM3II	AB297800	*Talaromyces purpureus* (L14527)	92% (480)
YM8-2	AB297802	*Hypocrea lixii* (AF443921)	100% (601)

[a]Code names that stand for the fungal isolates. [b]Accession numbers for the DNA sequences of the isolates. [c]The name of fungal species registered in GenBank that had a sequence with the highest homology to the DNA sequence of the isolates. Unidentified species were ignored. [d]The homology between reference sequences of the closest match and query sequences of the isolates.Total length of the query sequences are shown in parentheses.

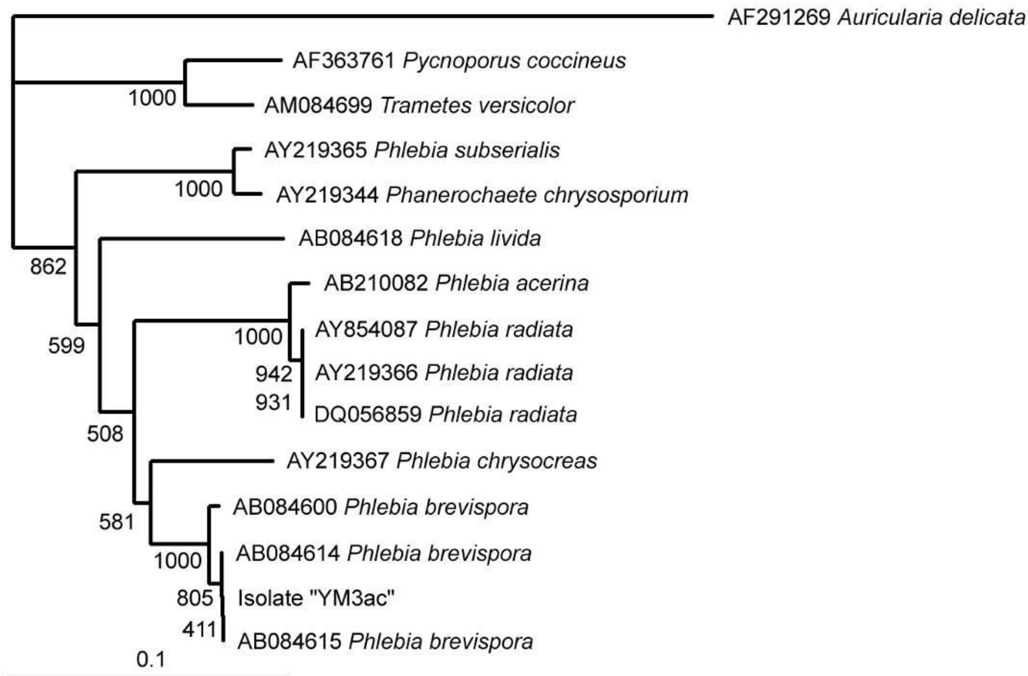

Figure 2. Neighbor-joining tree derived from the ITS sequences of a fungus isolated from the decayed xylem of *S. gibbosa* canker and reference strains from GenBank. Note the tree was rooted by the outgroup using the sequence of an Auriculariales fungus, *Auricularia delicata*. Bootstrap values higher than 500 out of 1000 are indicated. Bar stands for genetic distance (the number of nucleotide substitution per site).

The results of the BLAST search (Altschul *et al*. 1997) in the database showed the ITS sequences of the YM3ac isolate has 98% homology with *Phlebia brevispora* (AB084614) the closest matching species. The identification of the basidiomycete isolate was also supported by establishing a phylogenetic trees (Figure 2). The phylogenetic analysis based on the sequence using Clustal W revealed that fungus YM3ac was nested within the clade of *P. brevispora*. In this tree, the clade of *P. brevispora* was supported by a high bootstrap value, 805/1000.

In most cases, there was agreement concerning assignment to the species level when pairwise similarity scores from BLAST searches were over 97.9% (Kim *et al*. 2005). Therefore, based on the phylogenetic analysis and

the closest matching basidiomycete from the databases with over 98% sequence similarity were conspecific to our final identified fungus (Table 1). It was strongly suggested that fungus YM3ac belong to the species *P. brevispora*.

The identified decay fungus detected in this study has previously reported with no fruiting bodies (Erwin *et al.* 2007). This species might prefer decayed xylem, and would thus rarely form fruiting bodies in the forest. This fungus commonly known as white-rot decayer has also been isolated from utility poles (Nakasone *et al.* 1986) and butt rot of *Chamaecyparis obtusa*, Japanese cypress (Suhara *et al*. 1998).

The remaining fungi are all ascomycetes. These do not have xylem-rotting capabilities in either of the meranti trees though they may have role in stimulating the rate of decay (Blanchette and Shaw 1978)

Conclusions

By phylogenetic analysis based on the sequences of the ITS region of rDNA, basidiomycete fungi isolated from the decayed xylem of *S. gibbosa* canker were identified as *P. breviospora*. This is the first report of the decay fungus infecting *S. gibbosa*. The results of this study could be a useful reference in further pathological studies of *S. gibbosa*.

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