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# Characterization of *Rigidoporus microporus* isolated from rubber trees based on morphology and ITS sequencing

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White root disease of rubber trees caused by *Rigidoporus microporus* was studied the morphological and molecular phylogeny. The colony on PDA was white and flattening. The hypha showed hyaline, septum, no clamp connection. The fruiting bodies were broad shape, leathery, orange–red–brown in color. The hyphal system was monomitic, hyaline, thick walled with septate without clamp connection. Basidiospores were globose, thin-walled, colourless, and smooth. The *Rigidoporus microporus* is characterized by simple septate generative hypha, globose to subglobose smooth spores, mono to dimitic hyphal system, and causing white rot. Parsimony analysis of the ITS sequence confirmed the *Rigidoporus microporus* is distincted from *Rigidoporus ulmarious* but similar correlated to each other. According to molecular study, this is firstly recorded to study *R. microporus* based on ITS sequencing in Thailand.

**Key words:** *Rigidoporus microporus*, molecular phylogeny, ITS, sequencing

## Introduction

White root disease of rubber trees caused by *Rigidoporus microporus* (Sw.) Overeem is the most well known destructive disease in rubber plantation in many countries for example India, Indonesia, Malaysia, Sri Lanka, Thailand, West and Central Africa (Jayasuriya and Thennakoon, 2007). *Rigidoporus microporus* persists on dead or live root debris for a long time. It forms many white, flattened mycelial strands which grows and extends rapidly through the soil in the absence of any woody substrate (Nandris *et al*., 1987). The root of healthy rubber tree can be infected by contact with a disease source, such as

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rhizomorphs, infected root, dead stump, or wood debris (Nandris *et al*., 1987; Guyot and Flori, 2002). It can result in substantial death of trees and sometimes losses of a whole stand (Guyot and Flori, 2002). The fruiting bodies of this fungus form at the collar of the dead stem which produce a large number of basidiospores but seems to have a limited role in dissemination of this disease (Nandris *et al*., 1987).

The identification of wood decay fungi has been based on morphology, substrate utilization, and reproductive structures. This method is complicated because the fruiting bodies of wood decay fungi are frequently absent or difficult to detect in culture and also often show wide ranges of variability in physiologic characteristics, appearances and abilities (Hibbett and Donoghue, 2001). The morphological descriptions are used to identify cultivars; however, this approach lacks objectivity and reliability (Moukhamedov *et al*., 1994). The advances in molecular technology appear to offer a rapid method to identify fungi base on more objective evaluations (Diehl *et al*., 2004). The molecular techniques are already being used to identify of unknown species, genetic variability, characterization and relatedness of fungal isolates and species and pathogen detection (Wang, *et al*., 2005; Glen, 2006). The internal transcribed spacers (ITS) have been used widely in study on the relationships among species within a single genus or among infraspecific populations and study characterization in fungi (Hibbett and Donoghue, 2001). The ITS region is useful study for characterization in fungi for four reasons as follows:-1) the ITS region is relatively short (500-800 bp) and can be easily amplified by PCR using universal single primer (White *et al*., 1990), 2) the multicopy of the rDNA repeat makes the ITS region easy to amplify from small or dilute DNA samples, 3) the ITS region may be highly variable among morphologically distinct species, and 4) PCR-generated ITS species-specific probes can be produced quickly (Bridge and Arora 1998). ITS region have been used for the characterization, identification and detection of *Verticillium albo-atrum* and *V. dabliae* (Nazar *et al*., 1991) and used to differentiate *V. tricorpus* from the other species of *Verticillium* (Moukhamedov *et al*., 1994).

Although white root disease of rubber trees has been recorded as an economically important disease in Thailand, but it lacks of information on the genetic data. The objective of the study was to characterize of *R. microporus* by morphology and ITS sequencing.

## Materials and methods

### *Fungal isolates and morphological characterization*

*Rigidoporus microporus*, causing agent of white root disease of rubber tree was isolated by tissue transplanting technique from infected roots and fruiting bodies which obtained from the southern part of Thailand. The cultures were maintained in potato dextrose agar (PDA) medium and deposited at Biocontrol Research Unit, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. The characteristic of the fungus on PDA medium was studied such as growth rate, colony colour, the characteristic of hypha and other specific structure**s**. The fruiting body of this fungus was also studied by culture in plastic bag containing sterile inoculum mixed substrate which consisted of 100 g sawdust, 3 g rice bran, 2 g sugar for 30 days. The plastic bag was removed and covered the inoculum mixed substrate with soil, watered every day until fruiting bodies were produced. The morphology of fruiting bodies such as shape, color, and basidiospores was studied.

### *DNA sequencing*

***DNA extraction:*** Hyphal tip isolation was grown on potato dextrose agar (PDA) and incubated at 25 $^{o}$C for 10 days. The mycelium was scraped from the surface of PDA by aseptic technique. DNA was extracted with CTAB buffer as described by Kaewchai *et al*. (2009) by grinding mycelium with liquid nitrogen to fine powder. The mycelial powder was suspended in 600 µl Cetyltrimethylammonium bromide (CTAB), incubated at 65$^{o}$C for 30 min. During incubated period, the tube was vortexed every 10 min. After that the tube was cooled for a few minutes then 600 µl chloroform : isoamyl alcohol (CIA; 24:1, v/v) was added, gently mixed and centrifuged at 7,000 rpm for 5 min at 4 $^{o}$C. The aqueous layer was transferred to new tubes and extracted again with CIA. Finally, DNA was precipitated by adding 300 µl isopropanol, mixed well and incubated at room temperature for 30 min and centrifuged at 12,000 rpm for 10 min. The supernatant was decanted. The DNA pellet was suspended in 40 µl ddH2O.

***PCR amplification:*** The ITS regions were amplified using the universal primers ITS 1 (5′TCC GTA GGT GAA CCT GCG G 3′) and ITS 4 (5′TCC TCC GCT TAT TGA TAT GC 3′) (White *et al*., 1990). The amplifications were performed in reaction volumes of 50 µl containing 5 µl of 10x PCR buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 0.3 µl of 1.5 U *Taq* DNA

polymerase, 1 µl of each primer, 1 µl genomic DNA and 37.7 µl of $ddH_2O$. PCR was carried out in a MyGene$^{TM}$ Series Peltier Thermal Cycler (Model MG96G) using the following program: 2 min initial denaturation at 94 $^{o}$C followed by 35 cycles of 50 sec denaturation at 94 $^{o}$C, 1 min annealing at 55 $^{o}$C, 1 min extension at 72 $^{o}$C, and final extension for 5 min at 72 $^{o}$C. Amplification products were separated by 1% agarose gel in 1xTAE buffer strained with ethidium bromide which included in the agarose gel and visualized under UV fluorescence. PCR products were purified using the AxyPrep$^{TM}$ DNA Gel Extraction Kit (Axygen Scientific, Inc. USA) according to the manufacturer's instructions. DNA was sequenced by Shanghai Invitrogen Biolotech Co., Ltd. (Shanghai, P.R China).

***Data analysis:*** The ITS sequence from an isolate and related species retrieved from GenBank (Table 1) were included in the analysed. *Auricularia delicata* was set as out group. All sequences were edited and initial aligned by BioEdit, version 7.0.2 (http://www.mbio.ncsu.edu/BioEdit/bioedit. html). Multiple sequence alignments were determined with the ClustalX version 1.83 program. Gaps were set as missing data. Phylogenetic analyses were performed from aligned sequences of data sets using PAUP 4.0 version 4.0 Beta 10 (Swofford, 2001). Optimality criterion was set to parsimony. Bootstrap analyses were based on 1000 replications.

## Results

### *Morphological study*

The isolates of *Rigidoporus microporus* were studied on the morphology on the PDA medium and their fruiting bodies. The colony on PDA at room temperature (27-30 $^{0}$C) was circular in shape, white and flattening, grew full the PDA plate within 6 days with growth rate of 1.3 cm per day. The hypha showed hyaline, septum, no clamp connection, and possess many branches. The width of the hypha was vary from 2.8- 7.2 µm. The fruiting bodies were broad shape, leathery, and no stalk. The upper surface was orange–red–brown, smooth and the lower surface orange–brown, fine pores. In section, the hyphal system was monomitic, hyaline, thick walled with septate without clamp connection, hymenium with cystidioles. Basidiospores were globose, thin-walled, colourless, and smooth. The spore size was 3.6 - 4.1 µm (Fig. 1).

**Table 1.** Sequences from Genbank.

| Taxon | Strain N0. | Origin | Genbank accession |
|---|---|---|---|
| *Auricularia delicata* | | Costa | AF291290 |
| *Heterobasidion annosum* | wb276 | Austria | AF455496 |
| *Heterobasidion parviporum* | E1 | Latvia | FJ903330 |
| *Laetiporus gilbertsonii* | | USA | AM269785 |
| *Laetiporus* sp. | 6676 | Uruguay | EU840673 |
| *Laetiporus* sp. | 6677 | Uruguay | EU840674 |
| *Laetiporus* sp. | 6688 | Uruguay | EU840675 |
| *Laetiporus* sp. | 6689 | Uruguay | EU840676 |
| *Laetiporus* sp. | 6692 | Uruguay | EU840677 |
| *Laetiporus* sp. | 6693 | Uruguay | EU840678 |
| *Laetiporus* sp. | 6730 | Uruguay | EU840681 |
| *Laetiporus* sp. | 5179 | Uruguay | EU840682 |
| *Laetiporus sulphureus* | TENN61397 | USA | FJ596806 |
| *Oxyporus corticola* | R-3714 | USA | EF011122 |
| *Oxyporus corticola* | R-3713 | USA | EF011123 |
| *Oxyporus corticola* | 5385b | Estonia | DQ873641 |
| *Oxyporus corticola* | C70 | Latvia | FJ903327 |
| *Oxyporus cuneatus* | | Canada | DQ384575 |
| *Oxyporus latemarginatus* | I239 | Latvia | GU062267 |
| *Oxyporus latemarginatus* | CTM10133 | Tunisia | DQ000295 |
| *Oxyporus populinus* | R-3716 | USA | EF011121 |
| *Oxyporus subpopulinus* | 2313 | China | FJ644281 |
| *Oxyporus subpopulinus* | 2251 | China | FJ644282 |
| *Rigidoporus ulmarius* | | England | AY593868 |

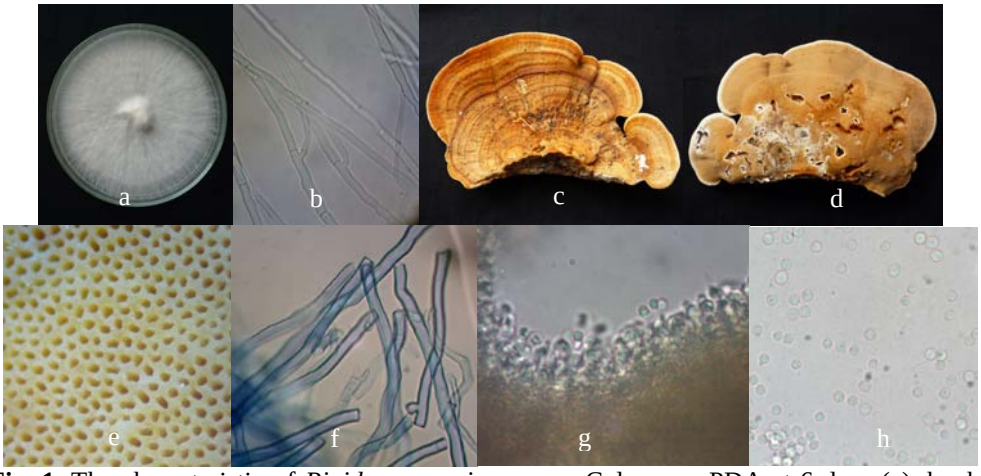


**Fig. 1.** The characteristic of *Rigidoporus microporus*. Colony on PDA at 6 days (a), hypha (b), fruiting body: upper surface (c) and lower surface (d), pores at the lower surface (e), monomitic, generative hypha (f), hymenium (g) and basidiospores (h).

### *DNA sequencing*

The final alignment of the ITS sequences comprised 32 taxa with 607 characters, 280 characters were constant expressed and 31 variable characters were parsimony-uninformative. The number of parsimony-informative characters was 296. The maximum parsimony (MP) heuristic search treating gaps as missing data with no differential weighting of transitions against transversions was studied using random addition sequence and tree-bisection-reconnection (TBR) branch-swapping algorithm resulted in 1 tree. When a weighted parsimony was applied to the same data set, with alignment gaps treated as missing data, the results also yielded 1 tree with consistency index=0.806, retention index=0.948, rescaled consistency index=0.764 and homoplasy index=0.194.

Parsimony analysis of the ITS sequence alignment resulted in one of the most parsimonious tree. Tree major groups were detected. The first group consisted of isolate of *Oxyporus* species. The second group consisted of *Rigidoporus microporus* and *R. ulmarious*. The third group was consisted of *Laetoporus* species (Fig. 2).

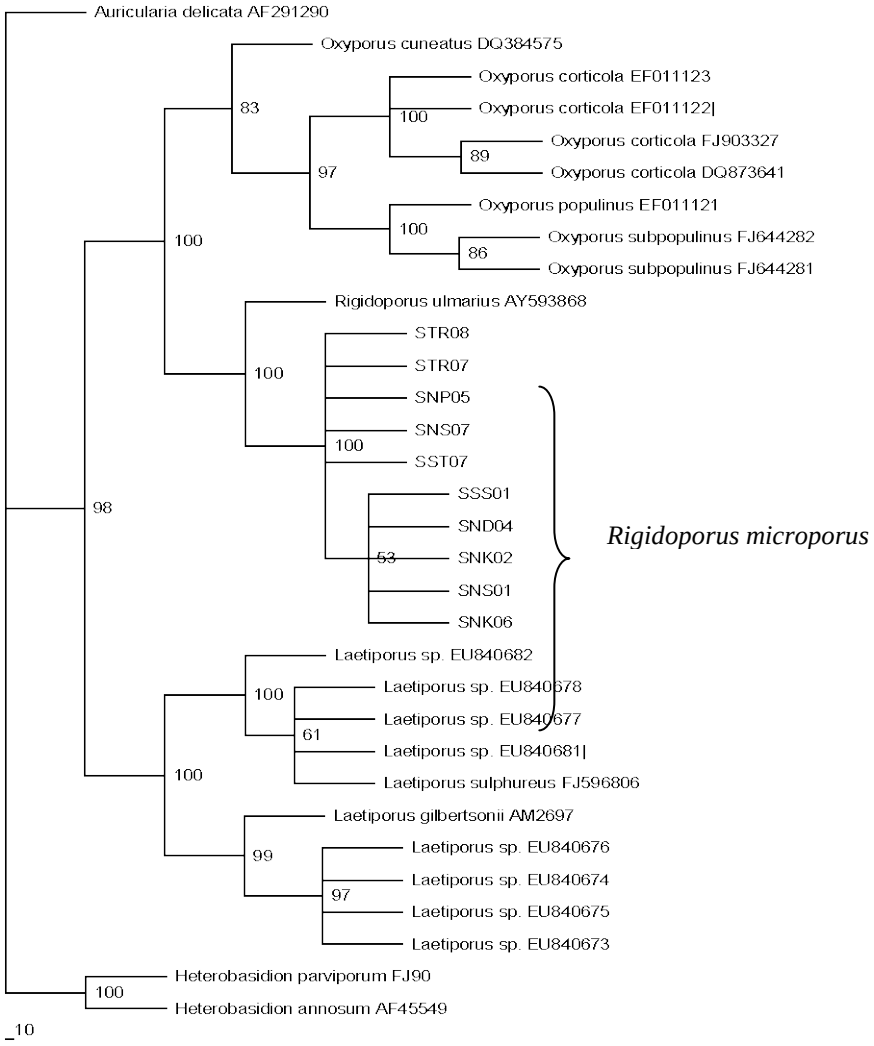


**Fig. 2**. Tree derived from maximum parsimony analysis of the sequences of the ITS region. The number at the branches denotes the percentage of bootstrap values after 1,000 replications and bootstraps higher than 50%.

## Discussion

The colony *of Rigidoporus microporus* on PDA showed white and flat. The hypha of this fungus showed hyaline, septum, no clamp connection, and possess many branches. This result was similar to the report of Nandris *et al*. (1987) who stated that the fungus formed many white and flattened mycelium but the colony on malt medium formed superficial, white mycelial felt. In the soil, the mycelial strands or rhizomorphs grow rapidly and may extend several meters in the absence of any woody substrate and can infect the root of the healthy trees. Moreover, the root of the healthy trees are infected by rhizomorph growing from stumps, infected wood debris buried in the ground as well as by roots contacting those of a diseased neighbouring tree (Nandris *et al*., 1987; Guyot and Flori, 2002).

The fruiting bodies of *Rigidoporus microporus* were broad shape, leathery, and no stalk. The upper surface was orange–red–brown, smooth and the lower surface orange–brown, fine pores. In section, the hyphal system was monomitic, hyaline, thick walled with septate without clamp connection, hymenium with cystidioles. Basidiospores were globose, thin-walled, colorless, and smooth. The spore size was 3.6 - 4.1 μm. These characters were similar to those which reported by Hood (2006) who reported that fruiting bodies a broad (to 20 cm wide), relatively thin, annual to less frequency perennial, leathery, broadly attached shelf, clustered, often imbricate; upper surface, concentrically furrowed, initially orange-red-brown, faintly, velvety, later smooth, faded; lower surface bright orange-brown, eventually paling, pores fine (6-9 per mm). In section, context pale colored. Monomitic, generative hyphae thin- or thick-walled, with cross walls (septa), with out clamps, hyaline. Hymenium with cystidioles, basidiospores sub-globose to globose (3.5-4.5 x 3.5-4 μm), thin-walled, colorless, smooth.

The phylogeny study is still needed for confirmation the specie of *R. microporus***.** The phylogenitic tree by ITS showed that *R. microporus* was similarly correlated to *R. ulmaroius*. The others groups were *Oxyporus*, *Heterobasidion* and *Laetoporus* group. Ryvarden (1991) reported that the *Rigidoporus* is in the group as same as *Melanoporia*, *Nigrofomes*, *Heterobasidion*, *Oxyporus*, *Leucophellinus*, *Laetiporus*, *Flavodon* and *Irpex*. This group is characterized by simple septate generative hypha, globose to subglobose smooth spores, mono to dimitic hyphal system, binding hyphae absent, and causing white rot but this research finding is confirmed that *R. microporus* is distinct from *R. ulmarius*.

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