In the first page, reviewer A gives general evaluation to our paper.

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(A)-1 The arrangement and title of chapters follow ITA ... OK

(B)-1 The paper is appropriately titled (The title should be brief and enough informative, not including abbreviations) ... OK

(B)-2 Keywords follow ITA ... NO

(C)-1 The abstract is brief and enough informative ... OK

(C)-2 The abstract has an appropriate length as indicated in ITA (at most 200 words) ... OK

(D)-1 All the tables and figures are necessary for the paper and they are enough informative ... OK

(D)-2 The style, title and legend of the tables and figures are appropriate ... OK

(E)-1 Reference articles are appropriately indicated in the text with sequential numbers. The style of reference list follows ITA ... OK

(E)-2 Reference articles are necessary for the paper and they are enough informative. It is especially recommended to refer papers published in JWS or 木材学会誌 in the last two years ... NO

(F)-1 The paper is new to science ... OK

(F)-2 The paper is informative ... OK

(F)-3 The paper is credible, not containing wrong logic, computations or experiments ... OK

(F)-4 Scientific terms are appropriately used ... OK

(F)-5 The sentences are linguistically acceptable ... OK

(F)-6 The text is brief and enough informative ... OK

(F)-7 Other comments on the paper ... YES

(G)-2 This paper is publishable after corrected considering reviewer’s comments

About this paper

Wood decomposition in the natural environment has not been enough investigated and still contains interesting subjects to be studied. It is understandable that decomposition in nature is hard to describe, because complex factors are involved. I appreciate that the authors made a great effort to address this issue; however, I do not like to deduce the scientific conclusion only from one case study, especially because the tree species used in the present study is commercially important.

I saw the opinion of the authors became more understandable in the text, owing to the improvement of the paper: e.g., rearrangement of experimental results along with decomposition stages. I also appreciate the authors' efforts on improving figures and other things. Therefore, I think this paper is acceptable after the authors would consider and discuss about that the results were based on only one case study and there still remain some questions to be solved, though those results are in agreement with previous studies.

I am very sorry about that I have made a wrong comment on the writing style of scientific names in the first time, which may have confused the authors very much. Please correct the paper as shown in "other comments" described below.

Other comments

Keywords: The word "cell" is not informative enough. Change it to another keyword that would be more characteristic to this paper.

P4L3: About the sample tree, the authors stated "we described the situation of the sampling site as much as possible;" however, description in the text was still not informative enough. Since it was the only one sample in the study, it should be described in a little more details. I guess the authors may indicate the aim of the paper more clearly if they add, for instance, "it was one of the typical trees diseased with canker" and "such trees were frequently found in the *Shorea smithiana* stand in that region" to the text.

P6L15: …dNTPs → each dNTPs [Takemoto has already corrected it. See the other document attached.]

P6L15q: The PCR mixture seems to contain many other reagents. I recommend the authors to add "according to the manufacture's instruction" to the text if they did not use a formula special to this study. (If they did, all the ingredients and formula should be

described.) [Takemoto has already corrected it. See the other document attached.]

Abbreviation of scientific names: I am sorry that my first comments included a wrong instruction. Please correct the paper as shown below.

Wrong; *Schizophyllum commune* → *S. commune*,
*Shorea smithiana* → *S. smithiana*

Right; *Schizophyllum commune* → *Sc. commune*,
*Shorea smithiana* → *Sh. smithiana*

References: Thank you for referring papers from J Wood Sci; however, the authors are still recommended to refer those published in the last two years. Please consider about this point.

That's all. Thank you.

**To Reviewer A**

この度は大変ご多忙中のところ、懇切丁寧な査読をたまわり、著者一同感謝申し上げます。

まずもって、先生のご指摘の箇所も含め、不鮮明な写真の撮り直し、実験データの再チェック、本文の記述内容の推敲、等を行いましたため、再提出まで時間がかかりましたことをお詫び申し上げます。英文につきましても、最初の投稿論文の際に依頼したところが不十分であったため、今回は国際的な英文チェック会社に依頼して訂正を行いました。

第一著者の Erwin 君はインドネシア国東カリマンタン州の Mulawarumann 大学からの留学生であり、学部・修士課程におきましては森林樹病の立場から熱帯樹木の canker tree について研究を行ってきました。従いまして、供試木の選定、試料の採取につきましても、一般的な解析結果が得られるようその canker を代表する樹木を選び、慎重にサンプリングと実験を行ってまいりました。

このことから供試木のサンプリングが 1 本の樹木であるというご指摘はその通りですが、得られた結果は熱帯材の中でも利用の面からも重要なレッドメランチの立木腐朽についての現象を組織構造的に解析し、劣化を引き起こす菌を分離・同定し、さらにそれを実験室的に接種して生じた腐朽現象で確認、考察するという一連の研究として意味があるのではないかと存じています。

論文種別を「ノート」へ変更しては、というご指摘を頂いていますが、再度解析内容を検討し直し、正確な論述に書き改めました。また、腐朽ステージの検討や菌の同定など重要な事項についても整理し直しました。どうかこの点をお含みおき頂き、「一般論文」として再度ご検討頂きますようお願い申し上げます。先生もご存じのように、J Wood Sci では論文種別の変更は却下扱いとなり一からのスタートになります。第一著者は学位取得を目指して頑張っていますので、どうかご理解の程をお願い申し上げます。なお、もう一方の査読者からは②の判定を頂いています。

変更内容、訂正箇所等は以下に記載しますが、本人が日本語不十分なため、記載事項は英語—日本語をやりとりして共同執筆者とで書き上げました。このため、十分気をつけましたが一部日本語の表記に不十分なところがありますればご容赦下さい。

改めて、ご多忙中での論文審査に対して御礼申し上げます。

**INTRODUCTION**

**P3,L19.：本研究では腐朽材から単離した菌………**

P3L19. Paragraph third that describes a brief of molecular method with stress was deleted from Introduction.

この段落で分子生物学的手法について強調して述べていましたが、この部分は削除いたしました。ご指摘のように、腐朽材から腐朽菌を単離し、同定した、という簡単な表現に訂正しました。

**MATERIAL AND METHODS**

**P4,L9：サンプルとして用いた腐朽材は1本なのか………**

P4L9 & P4L14: Collections of wood samples from decayed stem of the decayed tree was described in the text (P3L6). We have no data of the height of the tree.

サンプルの採取は腐朽材1本から行いました。腐朽木の腐朽部より試料採取した旨、本文に記載しました。なお、樹高についてはデータがありませんでしたが、できるだけ採取林分の状況を明示しました。

**P4,L14：供試木の健全部（腐朽していない部分）………**

ご指摘のように採取部位の説明が不十分でしたので、供試木の健全部から採取した旨を正確に記載しました。

**P5,L9：MEA の pH が記載………**

P5L9 (P3L12 after correction) pH of MEA was described in the text.

MEA の pH を本文に記載しました。

In the text:

The medium used for isolating wood-decaying fungi was 2% malt extract agar (pH 5.5) (MEA, 20 g of malt extract and 15 g of Difco agar in 1000 ml distilled water) with 100 ppm of chloramphenicol (antibiotic), and 2% MEA with 100 ppm of chloramphenicol and 100 ppm of benomyl (a fungicide with broad-spectrum activity on ascomycetes).

**P5,L21～：DNA 抽出において、試料の量（菌糸重量など）………**

P5L21 (P4L23) Approximate weight of mycelium was described in the text. The amount of 2-propanol (P5L9) was also added to the text.

本文に菌糸重量を記載しました。また、2-propanol の量も本文に明記しました。

**P6,L6：遠心分離の条件は………**

P6L6 (P5L7) The description of the condition of centrifugation was corrected replacing “rpm” by “×g”.

rpm で表示していた遠心分離の条件を、加速度“×g”にて記述し直しました。

In the text:

Chloroform-isoamyl alcohol (200 µl; 24:1) was added to microtubes containing the incubated tissue and centrifuged (15,000 x g) at 4 °C for 20 min

**P6,L12：PCR mixture の配列は………**

P6L12 (P5L15) The formula of the PCR mixture was corrected replacing the amount of each component by its final concentration.

各成分の分量で表示していた PCR 反応液の組成を、終濃度にて記述し直しました。

**P6,L16：primer の配列は………**

P6L16 (P5L17) The 5’- and 3’-ends of the primers were clearly described.

プライマーに５’端および３’端を明記しました。

**P7,L4：”genetic analyzer”は一般名詞ではなく………**

P 7 L4 (P6L5) The description of the genetic analyzer was corrected as the reviewer A suggested.

“genetic analyzer”はご指摘のように表現が不正確でした。 表記を、お示しして下さった例にならい次のように書き改めました。

In the text:

DNA sequences were determined using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, CA, USA).

**P7,L4：each fungus とありますが………**

P7L4 (P3L17) For isolation, additional explanations were mentioned in the text (Materials and methods).

本文中に加筆しましたとおり、合わせて３０株が分離されていますが、塩基配列を決定したのはそのうちの代表的な株のみです。得られたアイソレイトはコロニーの性状・胞子の形態等でいくつかの morphotype に分類し、各 morphotype から１株を任意に選定して分子生物学的解析に供しました。このような主旨の説明を本文に付加しました(Materials and methods).

In the text:

Three small pieces of wood (dimension: approximately 5 x 5 x 5 mm) were cut from decayed and sound sites on the disk (Fig. 1b): decayed wood in the canker margin (A–C), decayed wood adjacent to the region of sound wood (D), and sound wood away from the region of decayed wood (E). All wood samples were aseptically transferred to culture media in Petri dishes and then incubated at room temperature (24 °C) for several days. A small piece of agar containing fungal mycelium was transferred separately from the margin of the colony growing from each of the wood samples onto 2% MEA containing 100 ppm of chloramphenicol plated in a Petri dish, to purify the cultures. The medium was amended with 100 ppm of benomyl to obtain selective growth of basidiomycete fungi. 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 liquid medium (20 g of malt extract in 1000 ml of distillated water) in test tubes and incubated at room temperature for 3–5 days. Prior to transferring the fungal isolate, the liquid medium was sterilized as previously described. The young mycelium was used for fungi identification based on the ITS sequence.

**P7,L11～：パラメータを細かく定義した………**

P7L11- (P6L11) We used default set of the parameters for the alignment and phylogenetic analysis. Since we thought the reviewer suggested that our previous sentences were described too much in details, we shortened it, and concisely explained that we followed the default.

アラインメントおよび系統解析のパラメータはデフォルトの設定をそのまま用いています。冗長であるとのご指摘と存じましたので、本文中では細かい設定にふれるのはやめ、デフォルトの設定に従った旨簡潔に述べるにとどめました。

**P7,L22：腐朽試験に用いた培地………**

P7L22 (P6L24). The composition of the nutrient solution was corrected.

培地の組成について修正し、追記いたしました。

In the text:

Three blocks were placed in each one of four glass jars containing a medium of 250 g quartz sand and 80–85 ml of nutrient solution (4.0% glucose, 0.3% peptone, and 1.5% malt extract) and inoculated with the liquid fungal culture of the isolated fungus.

**P7,L24：12 週間と３ヶ月………**

P7L24 (P7L1) “Three months” was deleted. Twelve-week incubation was used in the text.

「３ヶ月」の表現をやめ、本文では「１２週間」の培養期間に統一することとしました。

**RESULTS**

**Microscopic evidence of *S. smithiana* wood decayed in forest**

**Fig.2～6 によって、環境中から採取した *S. smithiana* の………**

We have improved the explanation of Figs. 2-6 (Figs. 2-9, after correction) in the text P7L9 – P8L15

ご指摘のように顕微鏡観察による腐朽結果の報告が系列だっていませんでした。まさしくご指摘の通りだと反省しています。文章表現を推敲し、分かりやすい表現に改めるとともに、同一現象を示す複数の写真についてはそれらの部位を明示するようにして、腐朽ステージに沿って修正しました

腐朽ステージに関係する記述が時々出てきます………

By using Figs.2-9 we described wood degradation features of *S. smithiana* wood decayed in the tree. We recognized the pattern of wood degradation, which was belonging to simultaneous decay type. Different stages of wood decay in degraded areas were also detected under LM and SEM (Please see the text P7L9 – P8L15). We also used Fig. 11-13 to show that the wood degradation caused by the isolated decay fungus in laboratory decay test has similar pattern to *S. smithiana* wood naturally decayed, especially at the early decay stage.

Figs. 2-9 を援用しながら腐朽のステージを記述したのは、本研究に用い

た立木における *S. smithiana* 腐朽材の分解の様子です。私たちは木材分解様式を確認しましたが、それは simultaneous decay type にあたるものでした。光顕および走査電顕による観察では、本文で記述した以外にいくつかの腐朽箇所で異なる段階の木材組織の分解状況も見出していますので、それらを総合的に判断して腐朽の進行段階を類推しました。後述の部分で、本研究で分離された腐朽菌が実験室での腐朽試験で引き起こした木材分解の様式が、自然条件での *S. smithiana* 腐朽材のそれと類似していた、とりわけ初期段階の腐朽にあたるものと類似していた、という結論を導くために、このような腐朽の進行プロセスをあえて論述しました。

**<u>本研究で用いた腐朽供試木………</u>**

Based on these results, the decay pattern observed in the *S. smithiana* wood tissues naturally decayed was simultaneous decay, which was shown by presence of pit erosion, bore hole formation, eroded cell wall and by cell-wall thinning. In simultaneous decay, the lignin, cellulose and hemicelluloses are almost uniformly degraded at the same time during all decay stages. In cell-wall thinning, due to decay that progresses centrifugally from the lumen towards the middle lamella and cell corners, the largest lignin accumulations at middle lamella and cell corners was completely removed at the late decay stage.

観察結果にもとづけば、立木で腐朽を受けた *S. smithiana* 材組織にみられた腐朽様式は simultaneous decay であると言えます。その根拠として、壁孔の侵蝕、穿孔の形成、細胞壁の侵蝕と薄壁化がみられたことが挙げられます。一般に simultaneous decay においては、リグニン、セルロース、ヘミセルロースの分解は、腐朽段階全体を通じて同時的にほぼ均一に起こるとされています。細胞壁が薄化する際には内腔から細胞間層や cell corners に向けて腐朽が進行するのですが、このために私たちの観察結果では、細胞間層や cell corners に見られるリグニンの主要な集積箇所は腐朽段階の後期になると完全に喪失していました。

**<u>P8,L7：using light and scanning…とありますが、どの顕微鏡写真か………</u>**

P8L7 (P7L11). All figures were mentioned in the text.

本文で論述しています内容について、それらが的確に該当写真のどれであるかが分かるように記述し、かつそれぞれの写真において本文がどの部分を指しているのか分かるように言及するようにしました。

**<u>P8,L7：腐朽木 Fig.1 に示しながら………</u>**

P8L7. The figure was made bigger than before and equipped by sketch. The decay appears to extend from outside to inside and also tangentially (P7L8).

図を大きくし、模式図を追加しました。この写真を見ますと、腐朽は外部から内部へと拡がっているようですが、接線方向への拡がりも見られます。

**P8,L13：Due to complete of…..とありますが、cell component とは………**

P8L13. Due to complete of all cell components….etc. This sentence followed the previous sentence that mentioned that (Please see the text P8L7–P8L12) " In transverse sections view, partial degradation of the cell wall had a channel-like appearance in some fibers and axial parenchyma cells, which connect neighboring cells. With increasing decay, these channels enlarged and join to form cavities, decaying whole parts of the cell walls and also middle lamella and cell corners" (Fig. 7 and 9).

Therefore in the sentence has meaning whole parts of the cell walls, also middle lamella and cell corners, so it did not mean vessels, fibers, and others cells.

該当部分は、

Due to complete of all cell components….etc. の部分は、直前の "With increasing decay, these channels enlarged and join to form cavities, decaying whole parts of the cell walls and also middle lamella and cell corners."

という記述に続いています。つまり、この部分で述べた all cell components とは、細胞壁全体、細胞間層および cell corners を意味するものであって、道管、繊維などの細胞を示すものではありません。文章表現を誤解を与えないように訂正しました。

**P8,L18：サフラニン－ファーストグリーンの染色………**

P8L18. (P8L5). In the text, we have mentioned cellulose and hemicellulose of remaining cell wall were stained green after double staining. The figure was changed with a clear one.

本文中に、残存する細胞壁のセルロースとヘミセルロースは二重染色で緑色に染色されますが、その旨を記述しました。仮道管の細胞壁が白く見えるのはセルロースの分解も進行して、呈色できない程度の段階に達していることによると推定されます。

**Fig. 9 の写真のクォリティ………**

Fig. 9.This figure was replaced with a clear one and relocated as Fig. 2.

この写真は、別の鮮明なものに撮りなおして変更したうえで、順番を入れ替えて Fig. 2 としました。

**Identification of decay fungi based on DNA sequence**

**P9,L18：腐朽材から単離されたのは only one species………**

P9L18. For isolation, additional explanations were mentioned in the text (Materials and methods) as mentioned above.

分離方法の説明のため、本文中(Materials and methods)に新たな記述を加えました。内容は先ほど述べたとおりです。

We examine not all but several representative isolates selected. Additional explanation of the procedure was described in the text.

また、本研究では、全アイソレイトではなく代表的なものをいくつか選んで解析しています。その方法について本文中に説明を書き足しました。

**P9,L19：ベノミルなしの培地を用いて………**

P9L19. We have corrected the text (P8L20) as follows: "However, when 2% MEA medium with chloramphenicol (without benomyl) was used, two species of non-decaying mitosporic fungi were isolated, and no basidiomycete was detected".

ご指摘のように表現が曖昧でしたので、以下のように本文を修正しました。「しかしながら、クロラムフェニコールを添加した２%MEA 培地（ベノミルを含まない）を用いた場合には、分生子形成菌２種が分離され、担子菌は検出されなかった。」

**P10,L2：Cluster W を使って系統解析………**

P10L2 (P9L3) The analytical result by Clustal W was added to the Results section.

Clustal W による解析結果を Results に追記しました。

In the text:

The fungus RM4ac isolate resided within the clade of *Schizophyllum commune* and *Schizophyllum radiatum*, a result supported by a high bootstrap value (973/1000) in the phylogenetic tree.

**Microscopic evidence of *S. smithiana* wood decayed by *S. commune* in laboratory**

**<u>12 週間の腐朽試験を行った結果。。。。</u>**

For decay test, we followed the standard of JIS soil block procedure, in which wood block samples are inoculated with decay fungi and incubated for 12 weeks. That was the reason why we set 12-week incubation period.

腐朽試験に際しては、標準的な JIS K1571-soil block procedure に従いました。この方法においては、試料材片に腐朽菌を接種したのち 12 週間培養をおこなうことになっています。培養期間を 12 週間に設定したのはこのような理由からでした。この JIS 法による木材の腐朽試験に関しては、従来多くの研究が報告されていて、それらの結果と対比する意味でも、規格に準拠して行いました。また、ここで用いた培養条件では培地と菌糸の生育状況等を判断する限りでは、これ以上に培養期間を延長させても腐朽は進行しないのではとも考えました。

After finishing the decay test, we found some literatures that indicated low potential of *S. commune* as decay fungus. For instance, Schmidt and Liese, and Abdurachim (reference No. 22&23) suggested that this fungus is a serious wood destroyer under natural conditions, especially in tropical regions, but causes little wood decay *in vitro* (laboratory decay test). This may suggest, as you commented, we could yield the same decay pattern as observed in the samples naturally decayed if we would conduct another decay test longer than 12 week.

Discussion に記述しましたが、Schmidt and Liese, and Abdurachim (reference No. 22&23)の文献にも示しましたように、本菌はとりわけ熱帯地域において、自然条件下で深刻な材質劣化をもたらしますが、インビトロ（室内での腐朽試験）では殆ど木材腐朽を引き起こさないことを示唆しています。また、これまでの研究例のなかには、*S. commune* はさまざまな樹種に対し 7 ヶ月もの培養期間のあとでさえ重量減少はあまり引き起こさなかった(0.5–6.8%)と報告したものもありました。

However, some previous studies (references No. 20, 22, 23) reported that *S. commune* caused low weight loss on various species (0.5–6.8%) after as long as 7-month incubation period, which seemed difficult for the first author of this study to follow; He has to complete this paper to acquire his PhD by the end of this fiscal year before his going back to his home country. Although we were not able to observe for a longer time, we believe that the results of our laboratory test are comparable to the decay type of the natural samples. As mentioned in the text, the wood decay pattern caused by the *S. commune* isolate was similar to that of the early decay stage of *S. smithiana* wood decayed in the natural samples.

このような事情で、より長い期間の観察は実施しませんでしたけれども、本研究の室内試験の結果は野外試料の腐朽型と十分比較可能であると私たちは考えています。本文中に述べましたとおり、本研究で分離された *S. commune* アイソレイトの引き起こした木材腐朽の様式は、野外試料における *S. smithiana* 腐朽材にみられた初期の腐朽段階と類似していました。

**<u>P10,L15：Fig.14 について、菌糸が接着している………</u>**

P10L15. Fig. 14 have been removed. The sentence of cavity formed in ray

parenchyma wall in the text was also deleted. In other ray parenchyma walls, the cavity was not observed. We only detected penetration of fungal hyphae into ray parenchyma wall via cell pits as that observed on axial parenchyma walls (Fig. 12)

Fig.14 は削除しました。本文中の「放射柔組織の壁に形成された空洞」という部分も削除しました。この図以外の部分の柔組織の壁には、このような空洞は観察されませんでした。放射柔組織の壁においては、軸方向柔組織に見られたのと同様に、壁孔からの菌糸の貫入のみがみとめられました。

**Fig.9,11,12 の重要性が低いように思われ………**

Fig. 9. This figure, which shows clamped hyphae (Basidiomycete fungus) penetrating cell wall, was used to confirm the presence of decay fungus in *S. smithiana* wood decayed in the tree. The figure was relocated as Fig. 2. This figure was also mentioned in the discussed (P11L12)

Fig.9 の図は、担子菌の特徴を示す「クランプ」を有する菌糸が細胞壁を貫通しているところを示しており、立木における *S. smithiana* 腐朽材中の腐朽菌との共通性を明示する上で重要と判断しました。この図は、Discussion においても取り上げました。

Fig. 11 and 12. These figures were used to clearly indicate that the *S. commune* isolate was able to cause slight decay on *S. smithiana* wood under the laboratory condition, whose pattern was similar to that found in the naturally decayed wood samples. These figures were used also in the discussion.

また、Fig. 11 および 12 の図を用いたのは、本研究の *S. commune* アイソレイトが室内条件下でわずかな腐朽を引き起こす能力を有していたこと、また、その腐朽様式が自然条件で腐朽を受けた試料材片に見られたものと類似していたことを明示するためです。Discussion においても、この図を取り上げました。

**DISCUSSIONS**

***S. smithiana* の腐朽は細胞壁が薄くなる cavity……….**

In simultaneous decay, cell wall thinning takes place at the intermediate stage, whereas cavity formation (as resulted of decaying whole parts of the cell walls and also middle lamella and cell corners) appeared in transverse sections at the late stage. Please see the text P7L12, where Schwarze et al (reference No. 1) defined the different stages of simultaneous decay.

In many previous reports and wood decay studies (e.g. reference No 1,11,12,16,17), simultaneous decay caused by white-rot fungi was indicated to have specific characteristics, i.e general cell-wall thinning, presence of pit erosion, bore holes

formation, as well as cell wall erosion (erosion channels, erosion troughs, elongated erosion in cell wall).

In our study, we found cell wall thinning (single head arrow in Fig.7) and channel erosion (black arrow in Fig. 7). The channel erosions were enlarged and joined together to form cavities, decaying whole parts of the cell walls and also middle lamella and cell corners. The coalition of localized removal of the extensively degraded areas resulted in many large voids that appeared in transverse sections of the decayed wood (Fig.9).

When we used 2% MEA with benomyl for fungal isolation, only one Basidiomycete fungus, *S. commune* (white-rot fungus), was present in whole decayed wood. Likewise, we also observed that only one decay pattern took place (simultaneous decay) on the entire of decayed wood. Therefore, we suggested that only one basidiomycete fungus caused one decay pattern on *S. smithiana* wood decayed in the tree used in this study.

We also confirmed the natural decay pattern was similar to that on *S. smithiana* wood decayed by the basidiomycete isolate (*S. commune*) in laboratory decay test. Under laboratory decay test with 12-week incubation time, we observed signs of decay, such as pit erosion (decay cause pit enlarged and opening), bore holes and erosion troughs. Although the erosion of the wood cell walls did not appear to be extensive, the observation via LM and SEM did help to reveal that the patterns of decay in *S. smithiana* wood caused by *S. commune* under the laboratory condition are similar to those of the early stage of simultaneous decay found in the naturally decayed *S. smithiana* wood.

一般に simultaneous decay においては、細胞壁の薄化はその中期に起こり、これに対して空洞形成は（細胞壁全体のみならず細胞間層および cell corners が腐朽分解された結果であることから）後期の段階にみられます。Schwarze *et. al* (reference No. 1)による simultaneous decay のさまざまな腐朽段階の定義を本文の中に引用して論述しました。

また、白色腐朽菌による simultaneous decay では、cavity 等の細胞壁の侵蝕（侵蝕孔、侵蝕溝、細胞壁における細長い侵蝕痕）のほか、細胞壁の広汎な薄化や、壁孔への侵蝕および穿孔形成の存在、といった特異な性状が見られるとする既報(*e.g.* reference No 1,11,12,16,17)や木材腐朽に関する研究例が数多くあることから、本観察結果とも強く一致しております。

本研究では、細胞壁の薄化（Fig.7 に矢頭で示した）や孔状侵蝕（Fig.7 に黒矢印で示した）が見られました。こうした侵蝕孔は拡大し、互いに融合して空洞を形成していました。このような場合、細胞壁全体だけでなく細胞間層および cell corners にいたるまでが腐朽分解されていました。腐朽の激しい部位は局所ごとに欠損していきましたが、これらが連結されることによって生じた大きな空洞部が腐朽材片試料の横断切片中に多数現れていました。

ベノミルを添加した 2% MEA を菌の分離に用いた場合、担子菌である *S. commume*（白色腐朽菌）一種のみが腐朽材部全体から検出されました。また同時に、ただひとつの腐朽様式(simultaneous decay)がその腐朽材部全体で観察されました。これらのことから、本研究で用いた立木における *S. smithiana* 腐朽材では、一種の担子菌のみによってひとつの腐朽様式が生じたものと考えています。

私たちはまた、本研究の試料に見られた自然条件下での腐朽様式と、本

研究で分離した担子菌アイソレイト(*S. commune*)による室内腐朽試験での腐朽様式とが類似のものであることを確認しています。たとえば、12 週間の室内腐朽試験の後に観察された、壁孔侵蝕（腐朽によって壁孔の拡大と開口が起こる）、穿孔、侵蝕溝などです。木材細胞壁の侵蝕はさほど激しくないように見えましたが、光顕および走査電顕で観察してみると、確かに、室内条件下で *S. commune* が引き起こした *S. smithiana* の木材腐朽の様式は、自然条件下で腐朽を受けた *S. smithiana* の材に見られた simultaneous decay の初期段階と類似のものであると判断しました。

**P12L10：*Trametes trogii*……..**

P12L10. (P11L7 after correction). White-rot fungus *Trametes trogii* was used in the text.

本文では”White-rot fungus *Trametes trogii*”に訂正しました。

**考察に引用する論文は………**

特に「考察」に引用する論文について基本的な文献以外は古いものは削除し、新しい文献を中心として引用して考察を行うことにしました。

**REFERENCE**

- **古くて関連の薄い文献を削除し………J Wood Sci の文献………**
- **成書を引く場合も………**
- **Reference の 7 の書式………**

We removed some references (including reference No. 7) and used new references from Journal of Wood Science. We also added page number for all references except for the reference No. 8, which is computer software.

引用文献の中から古いものをいくつか削除しました。
Journal of Wood Science から新たな文献を引用しました（文献番号 46 (2000), 48 (2002)）。

また、No.8 以外の引用文献すべてにページ番号を付記しました。なお、No.8 はコンピュータソフトですのでページ番号はありません。

**Table, Fig.**

**. Table 1 は単離菌のリストとその ITS………**

Table.1 The title was corrected following the suggestion of the reviewer A. The description of the homology data and its explanation in the legend were also corrected for an easier understanding.

ご指示に従い、タイトルを改めました。

In the text:

**Table 1.** List of the fungal isolates from the decayed wood of *Shorea smithiana* stem canker and fungal species deduced from their ITS sequences

また、ホモロジーの欄とその説明を、分かりやすいよう改変しました。

**・Fig. 全体：Legend の Bar の記述………**

Legend and Bar. We used the explanations of bars in the legends for each figure following the paper in JWS Vol. 53 (2) 2007: 108-113

Legend and Bar　各図の Legend でのバーの説明については、JWS Vol. 53 (2) 2007: 108-113 に掲載の論文の体裁に従いました。

**・Fig. 1:写真だけでは………**

Fig. 1. (Fig. 1b, after correction) We made a sketch from the figure.

より分かりやすくするために、Fig.1 に模式図を作成して追加しました。

**・Fig. 3：3 種類の矢印が用いられていますが、、、、**

Fig. 3. (Fig. 7, after correction) We improve the arrows to make the figure easy to be understood.

図中において、論述箇所がどこを指しているのか分かりやすくなるように矢印を描き改めました。

**・Fig. 5：”RC”のマークが大きすぎて………**

Fig. 5. (Fig. 9, after correction) We small-sized the RC mark.

“RC”の印を小さくし、樹脂道等の状況が見えやすくしました。

**・Fig. 9：写真のクォリティが………**

Fig. 9. (Fig. 2, after correction) We changed the figure into another clear one

写真を再度撮り直し、もとの写真を別の鮮明な写真に置き換えました。

**・Fig. 10：印刷が粗く………**

Fig.10 The resolution was raised and the font size was enlarged. In the legend, we clearly described that bootstrap values higher than 500 out of 1,000 are indicated.

図の解像度を上げ、字を大きくしました。また、ブートストラップ値が 1000 回のうち 500 回以上のものを表示している旨、Legend に示しました。

**OTHERS**
その他

**P3,L9：*Shorea smithiana*………**

P3L9. It was corrected in the text P2L7

本文中の表記を、*S. smithiana* に揃えました**.**

**P3,L9：thedipterocarps……**

P3L9. It was corrected in the text P2L8

the dipterocarps に修正しました。

**P3,L25：一人称(we)を………**

P3L25. It was changed in the text P2L19

We を使う表現を避け、以下のように訂正しました。

In the text:

The present study aimed to (i) elucidate the microscopic features of naturally

decayed wood in a *S. smithiana* tree using light and scanning electron microscopy; (ii) identify the basidiomycete fungi and mitosporic fungi isolated from the decayed tissue based on the sequences of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA); and (iii) evaluate the decay potential and decay pattern of a basidiomycete isolate on *S. smithiana* wood in laboratory studies.

**P4,L2：the fungal decay……**

P4L2　The sentence including the words that the reviewer A had mentioned was totally changed.

ご指摘の箇所を含む文章全体を以下のように書き改めました。

**P5,L9：2% Difco malt extract agar…….**

P5L9. It was corrected in the text P3L12

本文を”2% Difco malt extract agar”のように修正しました。P3,L14 も同様に改めました。

**P5,L11：chloramphenicol の 2 回目の(antibiotics)は………**

P5L11. We deleted the second “antibiotics” from the text. We inserted “(fungicide which has a broad spectrum on ascomycetes)” next to “benomyl” in the text P3L15.

２回目の”antibiotics”を本文から削除しました。また、本文中の”benomyl”のあとに「(子嚢菌に対し広い活性を有する殺菌剤)」を挿入しました。

**P5,L12：単位として psi.を………**

P5L12. It was changed in the text P3L16

PSI.単位の使用を取りやめ変更しました。

**P7L7：URL に………**

P7L7 (P6L7) The word “BLAST” was deleted from the description of the URL.

”BLAST”を URL から削除しました。

**<u>P7L9：above 90%とした………</u>**

P7L9 (P6L9) We found some studies in which above-95% homology was used as the criterion for the identification of the genus. We corrected the text following this criterion. One of the representative papers that followed this criterion was sited as a reference.

関連文献にあたってみたところ、95%以上のホモロジーで同属と判定している例が複数見つかりましたので、その基準に沿って判断しましたが、その旨を本文中に記載し、この基準を用いている文献のうち代表的なものを引用しました。

**<u>P8,L5：*S. smitiana*…</u>**

P8L5. It was corrected in the text P7L7

本文の記載を、*S. smithiana* に修正しました。

**<u>P8,L7：from outside inward…..</u>**

P8L7. It was corrected in the text P7L8

本文の記載を、”from outside to inside”に修正しました。

**<u>P11L3：observed using LM and SEM…….</u>**

P11L3. We deleted LM and SEM and only used light microcopy and scanning electron microscopy in the text

本文中において LM および SEM の略記を取りやめ、”light microcopy”および “scanning electron microscopy”の表記で統一して用いることとしました。

**<u>P14,L1：*Schizophyllum*……..</u>**

P14L1. It was corrected in the text P12L24

*S. commune, S. smithiana* の表記に揃えました。Tf

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ORIGINAL ARTICLE

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**Miyuki Takeuchi · Takao Itoh · Yuji Imamura**

# Anatomical characterization of decayed wood in standing light red meranti and identification of the fungi isolated from the decayed area

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**Abstract** To further our understanding of wood decay in living light red meranti (*Shorea smithiana*) trees, microscopic characteristics of the cell and cell wall degradations of *S. smithiana* wood in the presence of the decay fungi, the identity of the causal fungi, and the decay potential and pattern by an isolated fungus were investigated. Cell wall degradations, including cell wall thinning, bore holes formation, rounded pit erosion, and eroded channel opening were clearly observed under light and scanning electron microscopy. In transverse view, many large voids resulting from a coalition of degraded wood tissue appeared in the decayed canker zone. All these observations suggest the well-known simultaneous decay pattern caused by white-rot fungi. By phylogenetic analysis based on the sequences of internal transcribed spacer region of ribosomal DNA, a basidiomycete fungus isolated from the decayed wood was identified as *Schizophyllum commune*. The degradation caused by this fungus on sound *S. smithiana* wood in an in situ laboratory decay test was classified as the early stage of simultaneous decay, and showed a similar pattern to that observed in the wood samples naturally decayed.

**Key words** *Shorea smithiana* · Wood decay · White-rot fungus · Phylogenetic analysis

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## Introduction

Because the decay of wood of living trees is a long-standing problem, the degradation of wood caused by decay fungi has been well characterized.[1] Nevertheless, such studies must continue because wood decay has not yet been investigated in many types of trees. One such example is basal stem canker of light red meranti (*Shorea smithiana* Sym.), a dipterocarp found growing naturally in Bukit Soeharto Forest in East Kalimantan, Indonesia.

*Shorea smithiana*, which grows in the lowlands of the Southeast Asian tropical rainforest, is a member of the Dipterocarpaceae family. Its timber is known as a member of the light red meranti group of timbers. The timber has been used in the manufacture of plywood, fiberboard, particleboard, molding, and other building materials. These species have also been established in large-scale plantation forests. 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.

Studying the microscopic characteristics of wood degradation and identifying the fungi involved in decay of the living tree of this commercial species will help to develop control measures against decay. Identifying the causal fungi and examining the decay pattern on wood caused by these isolated fungi in the laboratory should enable confirmation of the cause of degradation of the living wood.

The present study aimed to (1) elucidate the microscopic features of naturally decayed wood in a *S. smithiana* tree using light and scanning electron microscopy; (2) identify the decay fungi isolated from the decayed tissue based on the sequences of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA); and (3) evaluate the decay potential and decay pattern of the decay fungi on *S. smithiana* wood in laboratory studies.

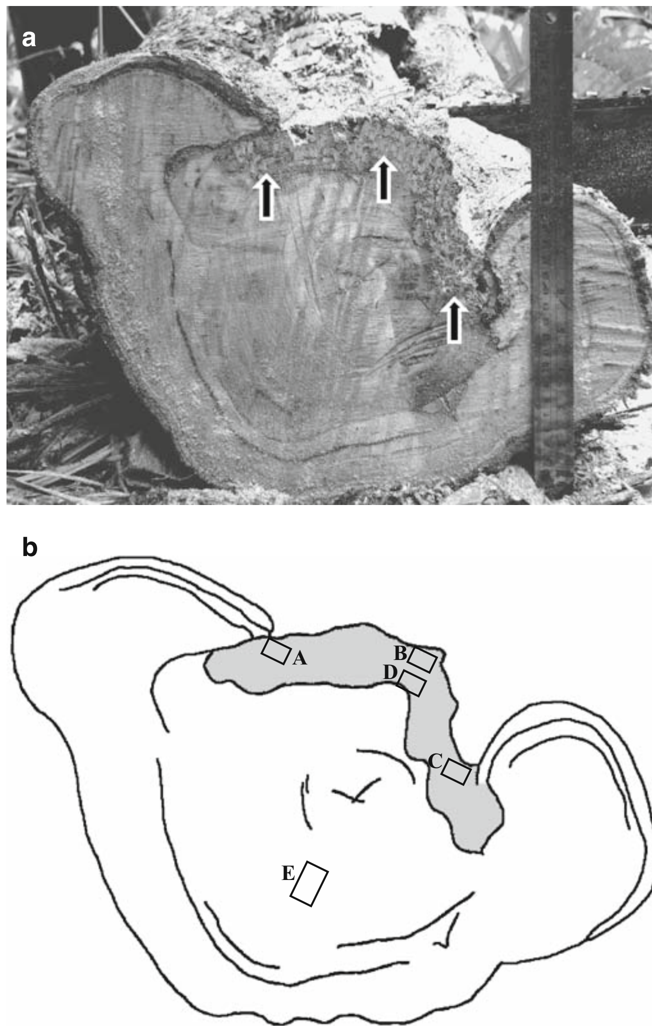


**Fig. 1.** **a** Cross-cut surface of *Shorea smithiana* log with exposed wood showing decay at the canker margin (*arrows*). **b** Sketch of **a** describing the zoning of the cut surface and the sites of sample collection. Decayed zone is *shaded gray*: *A–C*, decayed wood in the canker margin; *D*, decayed wood adjacent to the region of sound wood; *E*, sound wood opposite the region of decayed wood

## Materials and methods

### Wood sample collections

Wood samples were collected from a 45-year-old specimen of *Shorea smithiana* cankerous tree [diameter at breast height (DBH) approximately 55 cm] growing in a natural dipterocarp stand at the Bukit Soeharto Educational Forest of Mulawarman University, East Kalimantan, Indonesia. It was one of the typical trees diseased with canker, and such trees were frequently found in the *S. smithiana* stand in that region. The canker with decayed wood was formed on the stem from ground level up to 50 cm (Fig. 1). Four disks 5 cm thick were cut from the decayed stem. Three of these disks were used for microscopic observations of the anatomical characteristics of wood decay, and a fourth disk was used to isolate decay fungi. The sound, healthy portions were cut out from these disks and used for an in vitro laboratory decay test.

### Isolation of wood-decaying fungi

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

Three small pieces of wood (approximately $5 \times 5 \times 5$ mm) were cut from decayed and sound sites on the disk (Fig. 1b): decayed wood in the canker margin (*A–C*), decayed wood adjacent to the region of sound wood (*D*), and sound wood away from the region of decayed wood (*E*). All wood samples were aseptically transferred to culture media in petri dishes and then incubated at room temperature (24°C) for several days. A small piece of agar containing fungal mycelium was transferred separately from the margin of the colony growing from each of the wood samples onto 2% MEA containing 100 ppm of chloramphenicol plated in a petri dish to purify the cultures. The medium was amended with 100 ppm of benomyl to obtain selective growth of basidiomycete fungi. Finally, 30 isolates were established. 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 liquid medium (20 g of malt extract in 1000 ml of distilled water) in test tubes and incubated at room temperature for 3–5 days. Prior to transferring the fungal isolate, the liquid medium was sterilized as previously described. The young mycelium was used for fungi identification based on the ITS sequence.

### Microscopic observations

From each of the decayed stem disks, a wood sample (approximately $15 \times 15 \times 15$ mm) was taken from each of the five sites as mentioned above for fungi isolation (Fig. 1b). All wood samples were fixed in 2.5% glutaraldehyde, post-fixed in osmium tetraoxide, dehydrated in 50%, 70%, and 95% ethanol, each for 20 min, then three times in 100% ethanol, and embedded in Epon 812 resin. Transverse, radial, and tangential sections 20–50 mm thick were cut using a diamond knife, double-stained with safranin-fast green, and examined under a light microscope. For scanning electron microscopy, each of the wood samples was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C overnight, washed four times in 0.1 M phosphate buffer at pH 7.2 for 15 min each, and rinsed three times in distilled water for 5 min each. The samples were dehydrated in 50%, 70%, and 95% ethanol, each for 20 min, then three times in 100% ethanol. The dehydrated samples were

freeze-dried, mounted on stubs, and then coated with gold–palladium prior to examination with a Jeol Scanning Microscope (JSM-5310).

### 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 polymerase chain reaction (PCR) conditions generally followed the method described by Matsuda and Hijii,[2] which was modified from that of Gardes and Bruns.[3] The fungal mycelia were rinsed twice with CTAB buffer [50 mM Tris-HCl pH 8.0, 0.7 M NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% cetyltrimethylammonium bromide, 0.5% poly(vinyl pyrrolidone)]. CTAB lysis buffer (200 $\mu$l) was added to microtubes containing fungal mycelium and the tubes were subjected to three thaw cycles (from liquid nitrogen to hot water bath at 65°C). After the final thaw, the tissues were ground with a micropestle and incubated at 65°C for 45–60 min. Chloroform–isoamyl alcohol (200 $\mu$l; 24:1) was added to microtubes containing the incubated tissue and centrifuged (15 000 *g*) at 4°C for 20 min. After the supernatants were transferred to other microtubes, the DNA was precipitated by the addition of 200 $\mu$l of cold isopropanol and then by centrifugation. The DNA pellet was washed with 1 ml of 70% ice-cooled ethanol by centrifugation, then dried and resuspended in 60 $\mu$l TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

### PCR amplification

The ITS and 5.8S gene of rDNA of fungal isolates were amplified using PCR with total reaction volume of 25 $\mu$l (template DNA, ca. 0.5 ng/$\mu$l; each dNTPs, 200 $\mu$M; Taq DNA polymerase (New England Biolabs), 0.5 U; each primer, 0.5 $\mu$M, Tris-HCl, 1 mM; KCl, 5 mM). 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.,[4] 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.[4] The cycling condition and PCR temperature were adjusted following the method of Gardes and Bruns.[3] For checking DNA contamination of the reaction mixtures, negative controls (no DNA templates) were used. The amplified PCR products were then separated in 0.7% agarose gels and visualized using ethidium bromide. The PCR products were recovered from the gels and were manually purified as previously described.

### DNA sequencing and analysis

The purified PCR products were sequenced according to the method described by White et al.[4] 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/)]. The 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.[5] For basidiomycetes, further analysis was conducted using Clustal W version 1.83[6] 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[7] program in Clustal W based on Kimura-2-parameter distance. 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.[8]

### Decay test

Twelve sound wood blocks (20 × 20 mm in cross section × 10 mm in length) obtained from the uninfected portions of the stem disks (site E in Fig. 1b) were inoculated with the isolated fungus, and incubated in accordance with the JIS K 1571 soil-block test procedure.[9] The wood blocks were sterilized with gaseous ethylene oxide at 50°C for 5 h after oven drying and weighing. Three blocks were placed in each one of four glass jars containing a medium of 250 g quartz sand and 80–85 ml of nutrient solution (4.0% glucose, 0.3% peptone, and 1.5% malt extract) and inoculated with the liquid fungal culture of the isolated fungus. The four glass jars were then incubated at 26° ± 2°C and 70%–80% relative humidity for 12 weeks. In order to verify weight loss, nine blocks were oven-dried at 70°C until a constant dry weight was obtained; three blocks were reserved for anatomical observations.

## Results

### Microscopic evidence of *Shorea smithiana* decayed wood in the forest

A transverse view of the stem canker of *Shorea smithiana* shows what appears to be decay in the exposed wood at the canker margin. The decay appeared to extend from outside to inside and also tangentially (Fig. 1a, b). Examination by light and scanning electron microscopy on the decayed wood showed that various stages of simultaneous decay could be observed (Figs. 2–8). Schwarze et al.[1] noted that at the early stage of simultaneous wood decay, cell-wall degradation occurred in the immediate vicinity of the hyphae, and the cell wall was progressively broken down

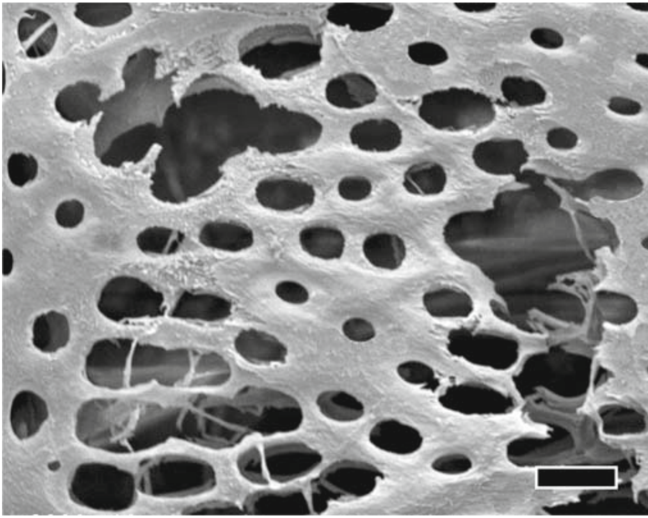

**Fig. 2.** Rounded erosion of intervessel pits, followed by the coalescence of the rounded pits. The sample was prepared from site *D* shown in Fig 1b. *Bar* 10 $\mu$m

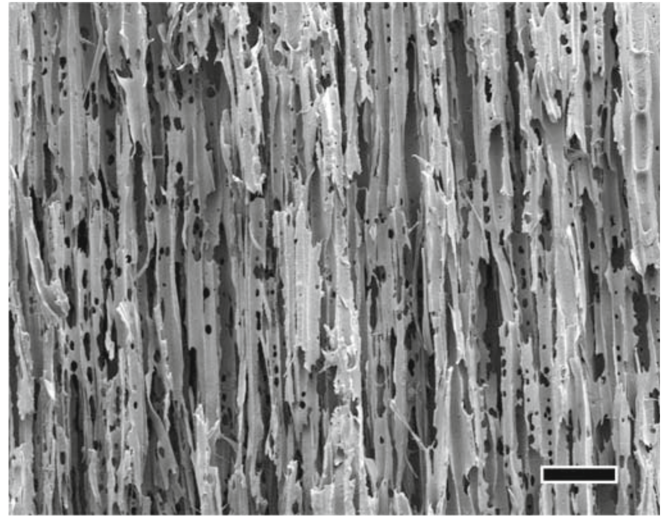

**Fig. 4.** Erosion troughs and numerous conspicuous holes were evident within fiber and parenchymal cell walls. The sample was prepared from site *A* shown in Fig 1b. *Bar* 100 $\mu$m

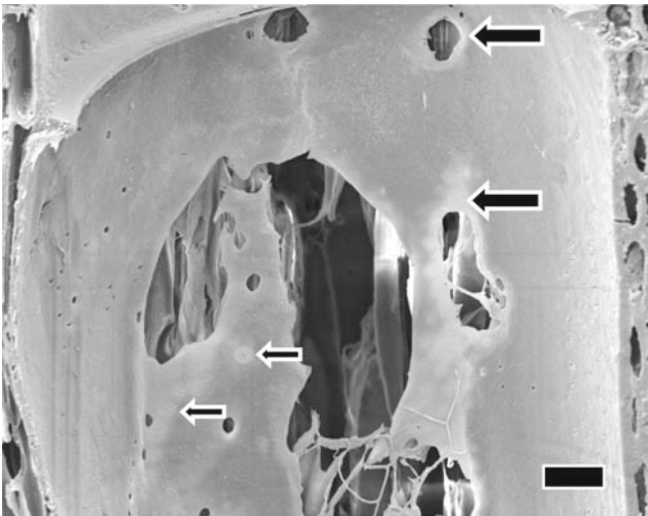

**Fig. 3.** Enlarged bore holes and axially elongated erosion troughs appeared in vessel walls. Lysis zones appeared around the bore holes and the erosion trough (*large arrows*), and as round spots (*small arrows*). The sample was prepared from site *D* shown in Fig 1b. *Bar* 20 $\mu$m

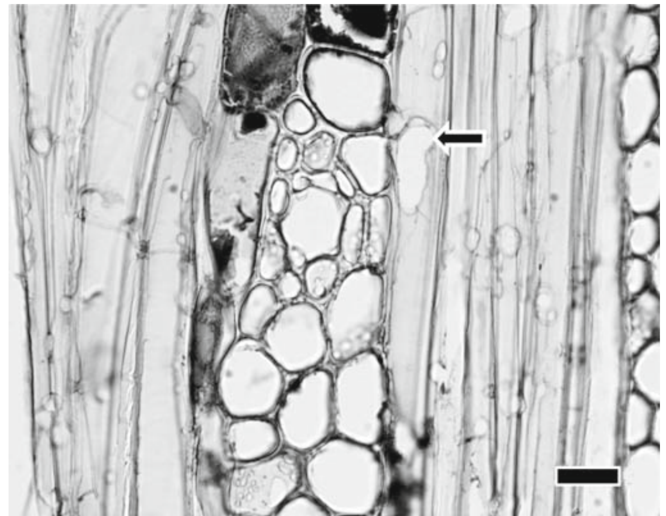

**Fig. 5.** Erosion channels with U-notches in fiber cell wall (*arrow*). The sample was prepared from site *A* shown in Fig 1b. *Bar* 20 $\mu$m

from the inside (cell lumen) to the outside; at the intermediate stage the cell wall became increasingly thinner, and numerous bore holes appeared between two neighboring cells At the late decay stage, the compound of middle lamella and cell corner degraded.

At the early stage of decay of wood cells, we observed basidiomycete hyphae colonizing the cell lumina and passing through cells via pit apertures with rounded pit erosion, bore hole formation in unpitted cell walls, and slight erosion of cell walls (Fig. 2). In vessel cell walls, as shown in Fig. 2, destruction of the intervessel pits occurred, appearing as rounded and distorted openings. Sometimes the intervessel walls were severely damaged. Figure 3 shows enlarged and coalesced bore holes, which then continued to be eroded until axially elongated troughs were formed.

At the intermediate decay stage, numerous and conspicuous holes with irregular rims were observed in fiber and axial parenchyma cell walls (Fig. 4). Fiber degradation was frequently observed in regions where ray parenchyma cells were in contact with degraded fibers (Fig. 5). Cell thinning could also be observed in some fiber and axial parenchyma cells, which resulted in wood cells becoming deformed (Fig. 6).

In transverse sections, the late decay stage was clearly detected, as shown in Fig. 6. Partial degradation of the cell wall had a channel-like appearance in some fiber and axial parenchyma cells, which were connected to neighboring cells. With increasing decay, these channels enlarged and

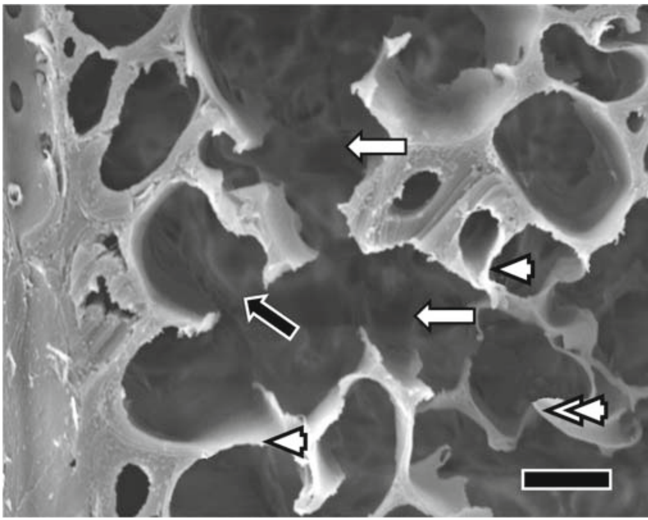

**Fig. 6.** Thinning (*single-head arrows*) and collapse (*double-head arrow*) of the cell walls, erosion channel (*black arrow*), and complete removal of cells (*white arrows*), which simultaneously occurred in one region of decay. The sample was prepared from site *C* shown in Fig 1b. *Bar* 10 μm

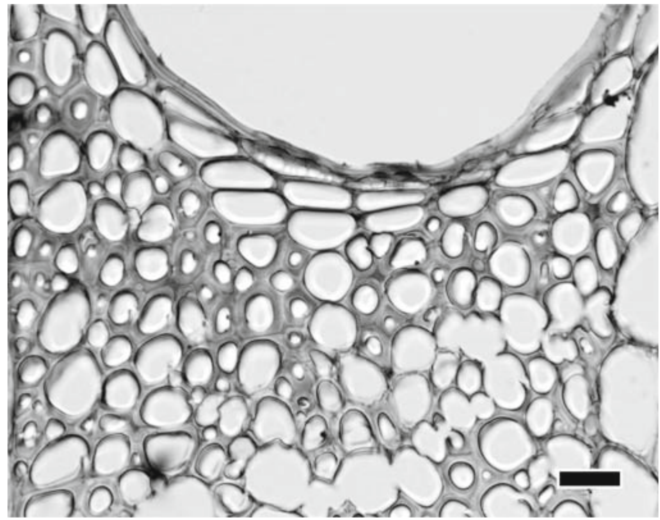

**Fig. 8.** Lignin remained in the cell corners and middle lamella, which were stained red by double staining with safranin-fast green at a progressive stage of decay. The sample was prepared from site *D* shown in Fig 1b. *Bar* 20 μm

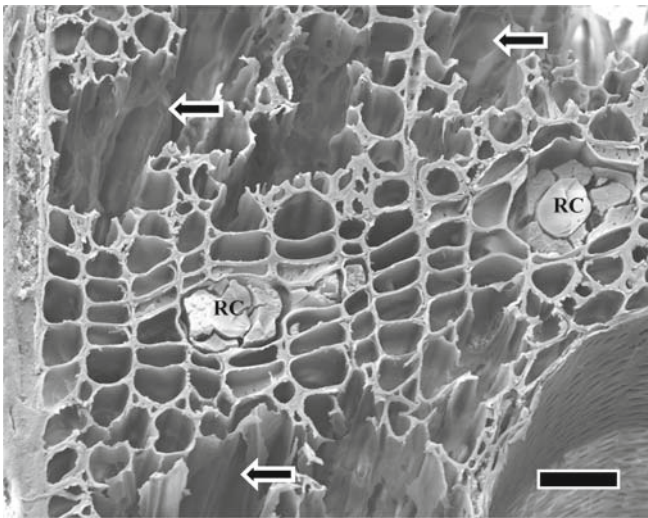


**Fig. 7.** Axial parenchyma cells adjacent to resin canals (*RC*) remained relatively unaltered. Note large voids (*arrows*) resulting from complete degradation of cell wall components within the degraded area. The sample was prepared from site *B* shown in Fig 1b. *Bar* 40 μm

joined to form cavities, decaying whole parts of the cell walls and also the middle lamella and cell corners. Due to the complete removal of all cell components, the remaining wood cells appeared to be separate from each other. Complete degradation of cell wall components resulted in many large voids that appeared in transverse sections of the decayed wood, as shown in Fig. 7. Advanced wood decay was also clearly observed in the severely degraded paratracheal parenchyma cells, while only axial parenchyma cells adjacent to resin canals remained (Fig. 7).

After double staining with safranin fast-green, light microscopy revealed that the middle lamella and cell corners with residual lignin were stained red, whereas the remaining fiber and axial parenchyma cell walls were mainly stained green, possibly due to the abundance of cellulose and hemicellulose (Fig. 8).

## Identification of decay fungi based on DNA sequence

Only one fungus was isolated from decayed tissue cultured on MEA with benomyl and chloramphenicol. This fungus had clamp connections on its hyphae, indicating that it was a basidiomycete. However, when 2% MEA medium with chloramphenicol (without benomyl) was used, two species of nondecaying mitosporic fungi were isolated, and no basidiomycete was detected.

When compared with sequences in the GenBank database, the ITS sequence of the basidiomycete isolate and the nondecaying fungi had more than 99% homology with the sequences of their closest matching species (Table 1). To complement the results of a BLAST search, we aligned the sequences of basidiomycete decay fungus (fungus RM4ac) with downloaded sequences from the GenBank database and performed phylogenetic analysis using Clustal W (Fig. 9). The fungus RM4ac isolate resided within the clade of *Schizophyllum commune* and *Schizophyllum radiatum*, a result supported by a high bootstrap value (973/1000) in the phylogenetic tree.

## Microscopic evidence of *Shorea smithiana* wood decayed by *Schizophyllum commune* in the laboratory

After 12 weeks of exposure in the laboratory decay test, wood blocks of *S. smithiana* that had been inoculated with *S. commune* fungus sustained an average weight loss of 1.8%. After initial colonization on the surface of *S. smithiana* wood, the hyphae of *S. commune* penetrated into the

**Table 1.** List of the fungal isolates from the decayed wood of *Shorea smithiana* stem canker and fungal species deduced from their internal transcribed spacer sequences

| Code name[a] | Accession number[b] | Closest match[c] (accession number) | Homology[d] (bps) |
|---|---|---|---|
| RM3 | AB297717 | *Penicillium daleae* (DQ132832) | 99% (510) |
| RM4 | AB297720 | *Trichoderma asperellum* (DQ109538) | 100% (411) |
| RM4ac | AB297718 | *Schizophyllum commune* (AF280758) | 100% (632) |

[a] Code names 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

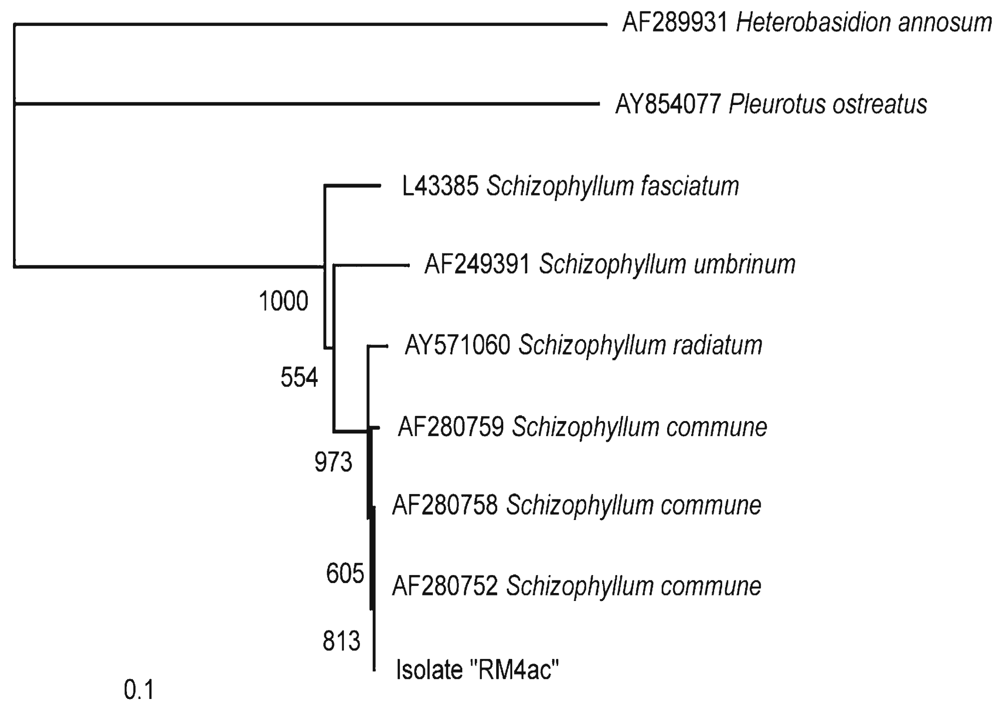


**Fig. 9.** Neighbor-joining tree derived from the internal transcribed spacer sequences of a fungus isolated from the decayed wood of *Shorea smithiana* stem canker and reference strains from GenBank. The tree was rooted by the outgroup using the sequence of an Aphyllophorales fungus, *Heterobasidion annosum*. *Bootstrap values* higher than 500 out of 1000 are indicated. *Bar* stands for genetic distance (the number of nucleotide substitution per site)

inner tissues. Hyphae appeared in the lumen of all cell types but were abundant mainly in vessels. The spread of fungal hyphae did not appear to cause extensive degradation of wood cells, and the intensive erosion and thinning of cell walls that occurred in decayed wood of the living tree were not detected. Nevertheless, signs of decay such as erosion troughs and pit degradation were frequently detected in infected cells. It appeared that hyphal branches penetrated to the vessel wall mainly through pits that caused distortion and enlarged openings (Fig. 10). Presence of erosion troughs in fibers and hyphae passing through pits were also observed in axial parenchyma cell walls (Figs. 11 and 12).

## Discussion

The presence of decayed wood at the canker margin indicating wound or injury was initiated by infection with decay fungi on the *Shorea smithiana* tree. With very few exceptions (e.g., wound parasites), it was shown empirically that most decay fungi preferentially infect and colonize the injured parts of the stem in which sapwood or heartwood is exposed.[1]

The three major types of wood decay are white, brown, and soft rots.[1,10] In white-decayed wood, two decay patterns have been well recognized: (1) simultaneous erosion, in which all wood cell wall components are degraded more or less simultaneously, and (2) selective delignification, in which lignin is broken down more than hemicellulose and cellulose. On the decayed wood of the living *S. smithiana* tree, the typical features of the simultaneous removal of all wood components were readily apparent. Degrading pits of cells, bore hole formation, thinning of secondary cell walls, and extensive cell wall erosion (Figs. 2–8), which are characteristic micromorphological features of simultaneous decay, were frequently detected in the present study. Similar patterns of wood degradation have also been recognized in other decayed broad-leaved trees, for instance, in beech trees.[1]

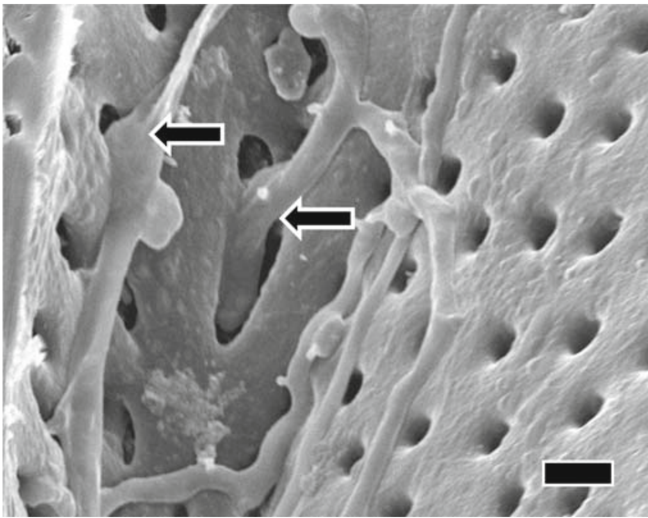

**Fig. 10.** Hyphal branches of *Schizophyllum commune* fungus passed through the vessel pits of *S. smithiana* wood after the laboratory decay test. *Bar* 5 $\mu$m

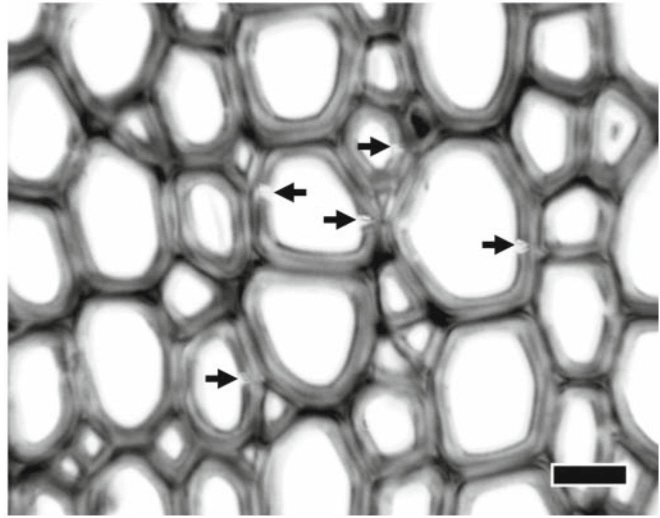

**Fig. 12.** Erosion troughs in fiber cell walls (*arrows*) of *S. smithiana* wood after the laboratory decay test. *Bar* 10 $\mu$m

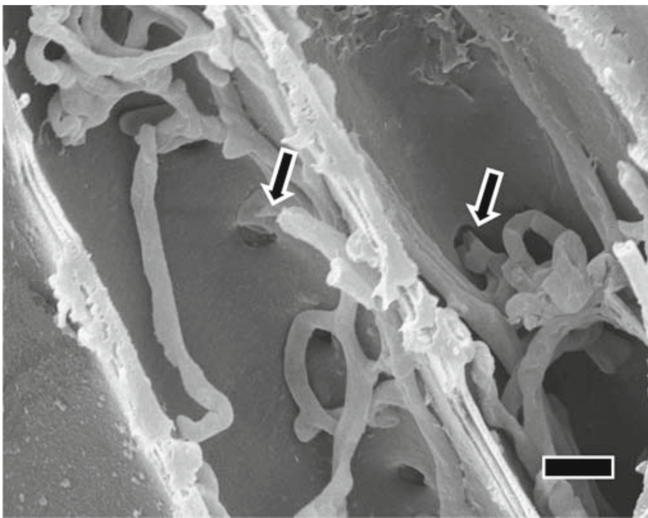

**Fig. 11.** *Schizophyllum commune* hyphae penetrated parenchyma cell walls through pits of *S. smithiana* wood after the laboratory decay test. *Bar* 5 $\mu$m

The presence of numerous holes and degraded pits in cell walls (Figs. 2 and 3) indicated that hyphae passed through cells both radially and tangentially, severely degrading cell walls. The subsequent erosion of cell walls is believed to be caused by enzymatic degradation.[11] Fungal enzymes that attack lignin, cellulose, or hemicellulose may be too large to diffuse into sound wood[12] and cause extensive degradation of wood elements not in direct contact with fungal hyphae.[13] The lysis zones that developed around bore holes, axially elongated troughs, and pits in cells, and which also appeared as round spots (Fig. 3), clarified the effects of fungal enzymes on cell walls, which were gradually eroded. The lysis zones indicated predelignification before the formation of actual bore holes, axially elongated troughs, and degraded pits. Liese[14] suggested that these simultaneous lyses of cell wall layers became progressively larger as the period of infection progressed, which might lead to large openings between cells. The erosion of the cell wall with axially elongated trough formation was also detected by Levin and Castro[15] in the simultaneous decay of poplar wood caused by the white-rot fungus *Trametes trogii*.

The characteristic decay pattern of cell wall thinning and channel openings were seen in the same regions of canker wood, as shown on Fig. 6, indicating that progressive decay proceeded from the secondary wall (cell lumen) toward the middle lamella. After progressive thinning of cell walls, the secondary walls were occasionally removed; only the middle lamella and cell corners remained. In channel formation, the compound middle lamella was apparently degraded and lost prior to opening. Cell degradation from the inside (lumen) to the outside has often been described as the pattern typical of simultaneous white rot.[1,16] Erosion channels with rounded edges (U-shaped notches) as well as enlargement of pit apertures were also readily observed in this study (Fig. 5). These typical aspects of the wood degradation were categorized by Anagnost[17] as simultaneous white rot associated with basidiomycete fungi.

The wood cells adjacent to axial resin canals remained intact (Fig. 7) probably because of terpenes, terpenoids, and polyisoprenes,[18] which are emitted from the resin canals and are toxic to decay fungi;[19,20] hence, those cells were locally protected from the invading fungi colonizing the vicinity of advanced decay regions.

It is interesting that only one basidiomycete species was isolated from the decayed wood tissue of *S. smithiana*. Based on the analysis shown in Fig. 9, the fungus RM4ac isolate resided within the clade of *Schizophyllum commune* and *Schizophyllum radiatum*. Furthermore, the fungus RM4ac had an ITS sequence identical to that of *S. commune* (AF280758). These results strongly suggest that RM4ac can be identified as *S. commune*. This fungal species, commonly

known as a white-rot fungus, has also been isolated from many decayed trees, for example, *Ocotea usambarensis*[21] and Chinese peach trees.[22]

The results of our laboratory decay test, in which *S. commune* induced weight loss of *S. smithiana* wood only slightly, were in accordance with previous studies that reported a weight loss of 0.5%–6.8% in various wood species after 2–7 months of incubation.[23,24] These findings are in agreement with the assertion of Schmidt and Liese[23] and Abdurachim[24] that this fungus is a serious wood destroyer under natural conditions, especially in tropical regions, but causes little wood decay in vitro. Laboratory conditions may not have been suitable for the fungus to extensively degrade wood despite colonization by the fungus. The degradation of wood in living trees may be due to the combined activities of many microorganisms. The presence of mold fungi such as *Penicillium* and *Trichoderma* in decayed wood could stimulate the rate of decay.[25]

The micromorphological changes in the cell walls of *S. smithiana* wood after 12 weeks of incubation provided important information about the pattern of the slight wood decay caused by *S. commune* under laboratory conditions. Light and scanning electron microscopy clearly revealed the hyphal penetration through the interelemental cell walls and their proliferation and colonization in the cell lumina, as well as the ability of this fungus to degrade the wood tissues.

Besides the longitudinal movement of hyphae in vessels, fibers, and axial parenchyma cells, and radial movement in ray parenchyma cells, hyphae colonized wood tissues by spreading from cell to cell via pit apertures and by penetrating across cell walls (bore holes). The degraded pits became enlarged without rupturing, suggesting that enzymes may be involved in the degradation of cell wall components around the pits. The presence of hyphae within the fiber S3 layers as well as in the erosion troughs of cell walls indicated that cell walls were progressively degraded from the cell lumen toward the middle lamella. These characteristics of wood cell degradation correspond to the simultaneous decay pattern characterized by white-rot fungi. When simultaneous decayers degrade wood tissues in vitro, erosion troughs are frequently detected in cell walls.[15,17] Although the erosion of wood cell walls did not appear to be extensive under laboratory conditions, light and scanning electron microscopy helped to determine that the patterns of decay in *S. smithiana* wood caused by *S. commune* were similar to those of simultaneous decay in the living *S. smithiana* tree.

*Schizophyllum commune* has not previously been reported to cause decay of *S. smithiana* trees. However, the presence of *S. commune* as a single basidiomycete fungus in the decayed wood of the *S. smithiana* tree, as well as its ability to produce in vitro a wood-decay pattern similar to that in a decayed cankerous tree, suggest that this fungus may be one of the causal agents of wood decay in *S. smithiana* trees. A further inoculation experiment is required to confirm the pathogenicity of *S. commune* to *S. smithiana.*

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