

**Molecular phylogeny of wood decay fungi of hardwood and their ability to produce laccase
that correlates with triphenylmethane dye decolorization**

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Abstract

Though Sri Lanka belongs to one of the 34 biodiversity hotspots of the world, its microflora specially fungi are not well studied and underrepresented in the global literature. Here we report the fungal species diversity of decaying hardwood of a Sri Lankan dry zone forest for the first time. Decaying hardwoods were collected from historically important Dimbulagala forest reserve, Sri Lanka and fungi associated with these woods were isolated. Out of 35 fungal species identified using morphological and molecular methods, 11 species were first records in Sri Lanka. All the tested isolates were able to utilize wood as the sole carbon source and produced varying degrees of laccase. Isolates of *Perenniporia tephropora*, *Coriolopsis caperata*, *Gymnopilus dilepis*, *Fusarium solani* and *Vanderbylia fraxinea* were among the top six laccase producers. Except *Fusarium solani*, the rest of the isolates showed more than 70% decolorization of the of triphenylmethane dye and there was a significant positive correlation between laccase production and dye decolorization. To the best of our knowledge laccase production and dye decolorization ability of *Vanderbylia fraxinea* and *Gymnopilus dilepis* have never been reported in the fungal kingdom before. *Perenniporia tephropora* was isolated from one of the strongest decay resistant hardwood species, Ebony (*Diospyros ebenum*) also known as dark wood and *V. fraxinea* was isolated from another medicinally important hardwood Neem (*Azadirachta indica*). Findings of this study confirms that decaying hardwood of Sri Lanka provide unexplored a unique niche for discovering fungal species with biotechnological applications such as high laccase producers and dye decolorizers.

Key words: Fungi, Hardwood, Laccase, Dye decolorization, ITS region

Introduction

Fungi are considered to be miniature metabolic factories having versatile secondary metabolites and enzymes with the potential to be used as work horses in diverse array of biotechnological applications [1]. Based on the Hawksworth's [2] estimation, there are about 1.5 million fungal species present on earth, which is considered as the baseline estimation. However, recent metagenomics studies suggested that actual numbers might be closer to 3.5 to 5.1 million species or much higher than this [3]. This uncertainty in the numbers is partially due to lack of advanced molecular based thorough studies in the tropics where incredibly rich diversity has been reported [4]. Hawksworth [5] also suggested that much of the undescribed fungal species could be present in the tropics and it is reviewed in Aime and Brearley [4].

Sri Lanka, a tropical island in the Indian ocean along with the Western Ghats, belongs to one of the 34 biodiversity hotspots in the world. Though its plant and animal diversity is well studied [6,7], microbial studies, especially fungal studies remain in its infancy. Furthermore, biodiversity hotspot concept of Sri Lanka should not be an exception for microbial diversity including fungi. However, most of the fungal studies in Sri Lanka have mainly focused on macro-fungi using morphological characters [8]. Moreover, these studies have mainly concentrated on the wet zone forests. On the other hand, dry zone forest ecosystems in the country spread over 22 % of area in Sri Lanka, whereas the total forest cover is about 26.6%. Dimbulagala (7°51'40.5"N 81°07'05.5"E) is an isolated hill covered with dry zone forest and it is rich with strong and economically important hardwood bearing plant species. This region is contacted with minimum anthropological activities for many years mainly due to 30-year long civil war in this region and was the study site of the current study. An unexplored niche, decaying hardwood, of

Dimbulagala forest reserve was selected with the aim of describing the species diversity of hardwood decay fungi for the first time in Sri Lanka.

For a healthy forest ecosystem, it is critical to recycle carbon stored in these hardwood and litter. Among many organisms such as beetles, flies, slime molds, bacteria, slugs and snails, primary decomposers that recycle carbon are fungi. It is known that wood, specially hardwood is a challenging substrate to degrade as it has very low nitrogen content (commonly C: N is about 500:1) and the presence of various fungal toxic compounds. Therefore, fungi specially basidiomycetes have evolved to degrade the hardwood structure consisted of cellulose, hemicellulose and the strongest natural polymer, lignin. They secrete major lignin modifying enzyme families such as lignin peroxidase, manganese peroxidase, versatile peroxidase and laccase [9]. Among them, fungal laccases have attracted the attention of scientific community due to its high redox potential and versatile catalytic properties compared to the laccases from other sources such as plants and bacteria [10]. Sri Lankan dry zone forests, including Dimbulagala forest reserve are rich in hardwood bearing plant species such as *Diospyros ebenum*, *Manilkara hexandra*, *Vitex pinnata*, *Diospyros chaetocarpa*, *Terminalia bellirica*, *Chloroxylon swietenia* and *Berrya cordifolia* [11–13]. These plant species produce hardwood with high economic value and are resistant to degradation. However, mycelial mats and mushrooms were often observed in decaying hardwoods in these areas indicating the involvement of fungal for the decay process. If that is the case, fungal species found in association with these decaying hardwood will be able to utilize wood as the sole carbon source. Therefore, the second objective was to assess the ability of the fungal isolates to grow on wood dust media. It was hypothesized that fungal species capable of growing on such hardwood have the ability to produce high amounts of laccases.

Fungal laccases have been identified not only as cell wall degraders, but also as strong oxidizers with the ability to oxidize di- and polyphenols, aromatic amines, and detoxify environmental effluents of food, paper, pulp and textile dyes [14,15]. Among these abilities of laccase, industrial dye decolorization has been the subject of many studies [16,17] and laccases are often considered as a “Green Tool” in biotechnology [18]. Basidiomycetes have often found to be high laccase producers as well as dye decolorizers. It was also intended to determine whether the isolates of the current study are capable of decolorizing an industrial dye, triphenylmethane with the hope of identifying novel dye decolorizers.

Out of tested 43 fungal isolates that could utilize wood as the sole carbon source, 11 were first records of Sri Lanka. Out of them two species were first time records as laccase producers and triphenyl methane dye decolorizers. There was a strong positive correlation between laccase production and dye decolorization.

Materials and Methods:

Sample collection

Sampling site was Dimbulagala historical forest reserve in Polonnaruwa district, Sri Lanka. Research permits were obtained from the authorities and samples were collected and handled ethically without harming living trees. Thirty-five decayed hard wood pieces of 5-6 cm length showing brown and white rot symptoms were collected randomly along the trail to the top of the hill (7°51'34.1"N 81°06'53.2"E to 7°51'42.8"N 81°07'09.0"E). Samples were collected into clean zip lock plastic bags and transported to the laboratory at University of Kelaniya (Fig. 1). Host of the decaying wood was recorded whenever possible. However, in most of the cases it was not

possible to clearly identify the plant species that decayed wood pieces belonged to. Samples were air dried for two days and stored in a refrigerator until further use.

Fig 1: Samples showing decaying hardwood (A) with mycelial growth and (B) white rot symptom.

Isolation of fungi into culture media

Decaying wood pieces (approximately 1 × 1 cm) were surface sterilized with 5% Clorox for 2 minutes followed by three serial washings with sterilized distilled water. Washed materials were blot dried using sterilized filter papers. Samples were trimmed from the edges and cut into approximately four pieces, placed on semi selective PDA media supplemented with streptomycin (100 µg/mL). Another isolation step was carried on semi selective medium slightly modified from Baumgartener et al. [19] by supplementing PDA with streptomycin (100 µg/mL) and fungicide carbendazim (4 µg/mL) to inhibit fast growing ascomycetes. Plates were incubated at room temperature for one week. All the fungal colonies originated from the wood pieces were sub cultured and pure cultures were obtained by hyphal tip isolation method. Pure cultures of 55 fungal isolates were obtained and vouchered both in sterile water and at -20 °C in Whatman number 1 filter papers at the Department of Botany, University of Kelaniya, Sri Lanka and assigned strain IDs.

Ability of isolated fungi to utilize wood as the sole carbon source

Ability of isolated fungi to utilize wood as the sole carbon (C) source was tested according to Swe [20]. During this assay most of the isolates those with morphological characters similar to

Trichoderma spp. were excluded and therefore, out of 55, 43 isolates were used in further studies. First, saw dust of *Mangifera indica* was passed through 2 mm sieve. Wood agar plates were prepared with saw dust (2% w/v), stock salt solution (5 % v/v), Hutner's trace elements (0.02 % v/v), with and without supplementing glucose (0.05% w/v) and agar (1.5 % w/v) followed by Swe [20]. Both wood agar plates with and without glucose were inoculated with 5 mm-diameter mycelial disc obtained from actively growing edge of 7-day old culture from each isolate. Plates were incubated at the room temperature (28 ± 2 °C) for three days. Colony diameters were measured in two-dimension perpendicular to each other. Experiment was repeated once.

Morphological and molecular identification of fungi

All the isolates were grown on PDA plates and used for morphological observation. Colony color, appearance and mycelial color, spores and sporulating structures when available of all the isolates were observed under phase contrast microscope (x 400 magnification) (Olympus CX41 model, Tokyo, Japan).

Out of all the isolates, 35 isolates that showed variable morphological features indicating putatively different species were selected for molecular level identification. Three mycelial plugs from actively growing colonies were inoculated into 50 mL Potato Dextrose Broth (PDB) supplemented with streptomycin (100 µg/mL). Flasks were incubated in a rotary shaker (Stuart-SSL1, UK) at 120 rpm at room temperature for 5-7 days. Mycelia were separated, washed with 500 µL of sterilized distilled water and squeeze dried with sterilized filter papers. DNA was extracted following modified method of Ceniz [21]. DNA Pellet was re-suspended in TE buffer (50-100 µL) and stored at -20°C until further analysis. Quality of extracted DNA was visually

observed on 0.8% agarose containing 0.5 µg/ml of Ethidium bromide. Polymerase Chain Reaction (PCR) was performed with universal ITS1 and 4 or 4 and 5 primer pairs [22]. Reaction mixture consisted of 1x Colorless GoTaq® Flexi Buffer, 2 mM MgCl₂, 200 µM each dNTP, 0.5 µM forward and reverse primers, 1.25U of GoTaq® DNA Polymerase (Promega Inc., USA) and <0.5 µg template DNA. PCR reaction was performed in a thermal cycler (Veriti® 96-Well Thermal Cycler, ABI Biosystems, USA) as published in Maduranga et al.[23]. Nuclease free water was added as the negative control and PCR products were separated using agarose gel electrophoresis and visualized under a Gel Documentation System (Quantum ST5, Germany). Pure PCR products were sequenced following Sanger dideoxy chain termination technology at the Genetech Institute, Colombo, Sri Lanka. Sequences were manually edited using BioEdit sequence Alignment Editor (Version7.2.5)[24]. Using Basic Local Alignment Search Tool (BLASTn), nucleotide sequences were compared with the authentic sequences available in the National Center for Biotechnology Information (NCBI) database and species were identified. All sequences were deposited in the GenBank and the accession numbers obtained are shown in Table 1.

Molecular phylogenetic analysis

DNA sequences were aligned using a multiple sequence alignment algorithm, Multiple Sequence Comparison by Log- Expectation (MUSCLE) [25], implemented in MEGA ver. X [26]. Gblocks 0.91b was used to eliminate poorly aligned positions and divergent regions of the alignment [27]. All characters were equally weighted and the gaps were treated as missing data. No out group was used and tree was unrooted. The evolutionary history was inferred using several phylogenetic tree construction methods, Maximum Likelihood, maximum parsimony and neighbor-joining, implemented in MEGA ver. X software. For Maximum Likelihood method, initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to

a matrix of pair-wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. To test the statistical support of each branch, one thousand boot strap replications were used as implemented in MEGA X.

Qualitative determination of laccase production by fungal spp.

PDA medium containing guaiacol was prepared by adding guaiacol at 0.01% w/v (G5502, Sigma) into PDA before autoclaving. Guaiacol PDA plates were inoculated using a mycelial disk (5 mm diam.) obtained from an actively growing edge of 7 days old culture plates. Triplicates of plates were incubated at room temperature (30-32 °C) for 5 days and the presence of brick red color around the mycelia or underside of the plate was observed [20]. Experiment was repeated once. Isolates that showed consistent red coloration were used for quantitative analysis.

Quantitative determination of laccase production by fungal spp.

Lignocellulose degrading enzyme production was quantitatively determined following [20] for selected 15 samples based on the qualitative assay. Liquid media consisted of 2 % (w/v) wood dust of *Mangifera indica*, glucose, salt solution and Hutner's trace elements as described in Swe [20]. Erlenmeyer flasks filled with 150.00 mL of liquid media (with saw dust) were autoclaved at 121°C for 20 min. and inoculated with 4 mycelial discs (5.00 mm diam.) obtained from the actively growing edges of 7-day old pure cultures of each fungal isolate. Experiment was conducted in triplicate for the selected 15 fungal isolates and one isolate continuously produced negative results was used as the negative control (Isolate IMF5). In addition, media with plain PDA plugs was also served as a negative control. Flasks were incubated at room temperature (30-32 °C) in a shaker

(Stuart-SSL1, UK) (120 rpm) and 1.00 mL of each sample was collected once in the 5th, 8th and 12th day during the incubation period. The collected samples were centrifuged at 5100 rpm for 15 min and the supernatant (here after referred as enzyme extract) was stored at -20°C [20]. Reaction mixture (5 mL) contained 3.90 mL acetate buffer (10 mM, pH 5.0), 1.00 mL guaiacol (1.76 mM) and 0.1 mL of the enzyme extract, and incubated at 25°C for 2 hrs. followed by the measuring of absorbance at 450 nm (Thermo scientific Multiskan GO, Finland). In the blank, enzyme extract was substituted with the buffer. Enzyme activity is considered to be corresponding with the increase in absorbance at 450 nm as indicated in Arora and Sandhu [28]. One-way Analysis of Variance (ANOVA) was carried out using Minitab 17 software (Minitab Inc., USA) to determine if there is a significant difference among isolates for the laccase production. Experiment was repeated once.

Triphenylmethane dye decolorization capacity of laccase producing fungi

Conical flasks containing 25 mL of sterilized medium containing glucose (10 g/L), KH₂PO₄ (1 g/L), MgSO₄ (0.5 g/L), CaCl₂ (0.14 g/L), yeast extract (1 g/L), thiamine (0.0025 g/L) and bromophenol blue (0.05%) were inoculated with 3 mycelial discs (5.00 mm) of 7-day old actively growing colonies [29]. Flasks were incubated at room temperature for 21 days in the shaker (Stuart-SSL1, UK) at 210 rpm. Two milliliters from each flask was obtained in 8th and 21st days after incubation and centrifuged at 12,000 rpm for 5 minutes to obtain cell free supernatant. Absorbance of each sample was measured at 590 nm (Thermo Scientific Orion aquamate 8000 UV-VIS spectrophotometer, USA) following Nidadavolu *et al.* [30]. Previously used IMF5 was used as the negative control. In addition, media with plain PDA plugs was also served as a negative control. The decolorization efficiency was determined using the following equation [31],

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$$225 \quad \% \text{ decolorization} = \left[\frac{A_{\text{initial}} - A_{\text{final}}}{A_{\text{initial}}} \right] * 100\%$$

226

227 where, *A initial* is the absorbance at the beginning and *A final* is the final absorbance.

228 To determine the correlation between laccase production and the dye decolonization abilities,

229 Pearson's correlation was carried out.

230

231 **Results:**

232 **Isolation of fungi from decaying woods:**

233 When PDA was used as the medium, very often fast growing genera of *Trichoderma* and

234 *Lasiodiplodia* spp. were isolated and therefore, semi selective media amended with both antibiotic

235 and benzimidazole was used to capture other genera as well. All together 55 isolates were obtained

236 and most of the colonies that showed *Trichoderma* like morphology were excluded from the

237 subsequent studies since it is well represented in many scientific literatures.

238 **Ability of isolated fungi to utilize wood as the sole C source**

239 None of the fungal isolates showed significant difference ($p < 0.05$) in their colony diameters on

240 glucose amended vs. glucose non amended media. In some instances, though not significant

241 glucose increased the colony diameters (Fig 2). All the tested isolates were able to utilize wood as

242 their carbon source and based on the colony diameters after three days of incubation, 43 isolates

243 were categorized into three categories as fast growers (> 6.0 cm diameter), intermediate growers

(6.0-3.0 cm) and slow growers (<3.0 cm). Figure 2 shows the variation in colony diameters on both media for the 43 isolates. Raw data were deposited in Dryad repository <https://doi.org/10.5061/dryad.g7f4m0j>

Fig 2. Bar chart showing mean colony diameter (cm) in wood agar (with and without glucose) and names of fungal isolates. Whiskers indicate one SE.

Identification of fungi

Since most of the fungal isolates were sterile (Fig 3) it was intended to use molecular identification method.

Fig 3. Colony morphology (10-day old culture in the left) and microscopic features of the isolates in the right of the isolates (A) KH2 and (B) DDW10A. Scale bar shows 50 µm and 20 µm respectively.

DNA extraction was successful for all the fungal isolates. When ITS1/4 or ITS4/5 primer combinations were used, most of the isolates produced single clear bands. Table 1 shows BLASTn results of each isolate along with the published/vouchered species that showed the highest sequence similarity. After removing most of the fungal cultures with morphologically similar characteristics on PDA, 35 were selected for molecular identification to avoid repetition.

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267 **Table 1: Isolate name, GenBank accession, species name and the GenBank accession**
 268 **number, which showed the highest sequence homology and the percent similarity.**

Number	Isolate name/voucher name	GenBank accession	Species (most similar GenBank accession)	Percent similarity
1	F1/DYF	MF164877	<i>Lentinus squarrosulus</i> (KT273373.1)	99%
2	F5/DBF	MF185103	<i>Daldinia eschscholtzii</i> (KY432354.1)	100%
3	F10/DHF5B	MF185104	<i>Fusarium decemcellulare</i> (MH857667.1)	99%
4	F9/DDW10A	MF185105	<i>Perenniporia tephropora</i> (JN048763.1)	99%
5	F4/DPW	MF185106	<i>Schizophyllum commune</i> (MF476007.1)	99%
6	F2/DSO	MF185107	<i>Hypoxylon</i> sp. (KC968924.1)	95%
7	F6/DZF4A	MF195026	<i>Coprinellus aureogranulatus</i> (MH379152.1)	99%
8	F7/IMF1A	MF195027	<i>Phanerochaete</i> sp. (HQ589222.1)	94%
9	F8/TW2AWP	MF195028	<i>Gymnopilus dilepis</i> (KT368680.1)	99%
10	F3/TW5	MF195029	<i>Scytalidium</i> sp. (AY762623.1)	86%
11	F11/DZFK2	MF195030	<i>Fusarium solani</i> (M277969.1)	98%
12	F12/DHF1	MF195031	<i>Hexagonia</i> sp. (KX900637.1)	81%

13	F13/DHF2	MF289184	<i>Perenniporia tephropora</i> (JN048763.1)	98%
14	F14/ADW2A	MF289185	<i>Coriolopsis caperata</i> (KR078279.1)	99%
15	F16/ADW3	MF289186	<i>Phanerochaete australis</i> (KP135080.1)	98%
16	ADB2	MF671942	<i>Trichoderma harzianum</i> (KT852806.1)	99%
17	ADB4	MF671943	<i>Lasiodiplodia crassisporea</i> (FJ888477.1)	98%
18	ADB5	MF671944	<i>Trichoderma harzianum</i> (KT852806.1)	99%
19	ADB7	MF671945	<i>Lasiodiplodia crassisporea</i> (HM466955.2)	99%
20	ADW5	MF671947	<i>Trichoderma harzianum</i> (KF201295.1)	99%
21	ADW8	MF671948	<i>Lasiodiplodia pseudotheobromae</i> (EU860391.1)	98%
22	DB8	MF671949	<i>Lasiodiplodia crassisporea</i> (JX464058.1)	99%
23	DW9	MF671950	<i>Trichoderma erinaceus</i> (MG372128.1)	99%
24	DW10	MF671951	<i>Lasiodiplodia pseudotheobromae</i> (MG870583.1)	98%
25	DW11	MF671952	<i>Phialophora alba</i> (HM116755.1)	99%
26	ADB2A	MF671989	<i>Trichoderma harzianum</i> (KT852833.1)	100%
27	ADB3	MF671990	<i>Xylogone sphaerospora</i> (GQ272626.1)	98%
28	DDW2	MF671991	<i>Neoscytalidium hyalinum</i> (MH861121.1)	99%
29	DDW5	MF671992	<i>Pleurostoma richardsiae</i> (MH859458.1)	99%
30	DDW6	MF671993	<i>Trichoderma longibrachiatum</i> (KJ174214.1)	99%

31	DDW10B	MF671994	<i>Paecilomyces formosus</i> (MH859718.1)	99%
32	DDW10C	MF671995	<i>Hypoxylon fragiforme</i> (KJ826511.1)	98%
33	DW7	MF671996	<i>Lasiodiplodia crassispota</i> (JX464058.1)	99%
34	KH2	MG890271	<i>Vanderbylia fraxinea</i> (KX081102.1)	99%
35	ADW1	MF671946.2	<i>Trichoderma harzianum</i> (KT852833.1)	99%

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270 Molecular phylogenetic analysis

271 The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-
272 Nei model [26]. Unrooted the tree with the highest log likelihood (-9113.58) is shown in the Fig
273 3. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join
274 and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite
275 Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value.
276 In 35 isolates, there were a total of 654 positions in the final dataset. The percentage of trees in
277 which the associated taxa clustered together is shown next to the branches for maximum
278 likelihood, Maximum parsimony and neighbor joining methods respectively. Colony diameter
279 data were also included and represented by +, ++ or +++ to indicate slow medium and fast
280 growth respectively.

281

282 **Fig 4. Maximum likelihood tree (unrooted) with the highest log likelihood showing**
283 **phylogenetic placement of 35 wood decay fungi isolated from Sri Lanka. Bootstrap support**
284 **is shown above each branch in three different analyses, Maximum Likelihood, Maximum**

Parsimony and Neighbor Joining respectively. Amount of laccase production in quantitative assay is depicted in red colored boxes in each taxon (darker the color higher the laccase production). Colony diameter is indicated in +, ++ and +++ to represent slow medium and fast growers respectively. Growth diameter was not determined for the isolates with empty cells.

Qualitative determination of laccase production by fungal spp.

Out of 43, only 15 isolates were observed to be consistently producing laccase as indicated by the production of dark red/brown color development in the PDA medium amended with guaiacol in several repeated experiments. Evaluated fungal strains produced varying levels of color intensities as well as coloration patterns while negative control remained without any red color development.

Quantitative determination of laccase production by fungal spp.

Isolates were significantly different from each other in their mean absorbance values as measured in 5th, 8th and 12th days indicating significant difference in laccase production ($p < 0.05$) (Table 2). Both of the isolates of *Perenniporia tephropora*, and the rest of the isolates of *Coriolopsis caperata*, *Gymnopilus dilepis*, *Fusarium solani*, *Vanderbylia fraxinea* and *Scytalidium* sp. were among the top seven laccase producers in both trials.

Table 2: Varying amounts of laccase production as indicated by the absorbance values and percentage dye decolorization ($\pm$ SD) of the isolates. Isolates that do not share the same letter

Isolate	Species name	Laccase production			Percentage decolorization of bromophenol blue	
		Mean absorbance values $\pm$ SD			Mean % decolorization $\pm$ SD	
		5 th day	8 th day	12 th day	8 th day	21 st day
DDW10 A	<i>Perenniporia tephropora</i>	0.158 $\pm$ 0.004 ^a	0.168 $\pm$ 0.006 ^a	0.166 $\pm$ 0.003 ^a	71.173 $\pm$ 0.974 ^a	89.29 $\pm$ 0.680 ^a
DHF2	<i>Perenniporia tephropora</i>	0.153 $\pm$ 0.006 ^a	0.152 $\pm$ 0.008 ^a	0.142 $\pm$ 0.009 ^b	70.800 $\pm$ 0.230 ^a	87.19 $\pm$ 0.506 ^b
ADW2A	<i>Coriolopsis caperata</i>	0.144 $\pm$ 0.006 ^{a,b}	0.157 $\pm$ 0.004 ^a	0.115 $\pm$ 0.008 ^c	70.087 $\pm$ 0.172 ^a	84.63 $\pm$ 0.569 ^c
TW2AW P	<i>Gymnopilus dilepis</i>	0.128 $\pm$ 0.009 ^{b,c}	0.124 $\pm$ 0.004 ^b	0.131 $\pm$ 0.006 ^{b,c}	54.753 $\pm$ 0.396 ^b	74.18 $\pm$ 0.742 ^c
DZFK2	<i>Fusarium solani</i>	0.122 $\pm$ 0.002 ^c	0.122 $\pm$ 0.005 ^b	0.121 $\pm$ 0.002 ^c	14.923 $\pm$ 1.236 ^f	15.11 $\pm$ 0.196 ⁱ
KH2	<i>Vanderbylia fraxinea</i>	0.117 $\pm$ 0.005 ^c	0.124 $\pm$ 0.001 ^b	0.139 $\pm$ 0.004 ^b	52.047 $\pm$ 0.365 ^c	79.07 $\pm$ 0.396 ^d
TW5	<i>Scytalidium</i> sp.	0.111 $\pm$ 0.010 ^{c,d}	0.133 $\pm$ 0.006 ^b	0.119 $\pm$ 0.008 ^c	11.427 $\pm$ 0.396 ^g	18.45 $\pm$ 0.879 ^h
DLP*	n/a	0.098 $\pm$ 0.006 ^{d,e}	0.085 $\pm$ 0.003 ^c	0.088 $\pm$ 0.003 ^d	39.683 $\pm$ 0.455 ^d	40.17 $\pm$ 0.172 ^f
ADW3	<i>Phanerochaete australis</i>	0.083 $\pm$ 0.007 ^{e,f}	0.119 $\pm$ 0.008 ^b	0.115 $\pm$ 0.005 ^c	11.463 $\pm$ 0.800 ^g	24.61 $\pm$ 0.287 ^g
DHF5B	<i>Fusarium decemcellulare</i>	0.081 $\pm$ 0.002 ^f	0.079 $\pm$ 0.007 ^c	0.066 $\pm$ 0.005 ^e	n/a	n/a
DPW	<i>Schizophyllum commune</i>	0.069 $\pm$ 0.003 ^f	0.074 $\pm$ 0.001 ^{c,d}	0.089 $\pm$ 0.004 ^d	12.627 $\pm$ 0.597 ^g	12.03 $\pm$ 0.919 ⁱ
DZF4A	<i>Coprinellus aureogranulatus</i>	0.050 $\pm$ 0.004 ^g	0.059 $\pm$ 0.006 ^{d,e}	0.086 $\pm$ 0.006 ^d	17.437 $\pm$ 0.469 ^c	18.53 $\pm$ 0.168 ^h
IMF1A	<i>Phanerochaete</i> sp.	0.047 $\pm$ 0.005 ^g	0.059 $\pm$ 0.007 ^{d,e}	0.039 $\pm$ 0.004 ^f	n/a	n/a
DBF	<i>Daldinia eschscholtzii</i>	0.045 $\pm$ 0.007 ^g	0.046 $\pm$ 0.003 ^c	0.044 $\pm$ 0.001 ^f	n/a	n/a

were significantly different from each other ($p < 0.05$).

* repeated attempts of DNA barcoding was unsuccessful n/a- not determined

IMF10*	n/a	0.040 ± 0.002^g	0.116 ± 0.003^c	0.112 ± 0.005^d	$13.227 \pm 0.902^{f,g}$	19.32 ± 0.363^h
IMF5*	negative control n/a	0.001 ± 0.005^h	0.004 ± 0.007^f	0.003 ± 0.003^g	01.100 ± 0.676^h	0.607 ± 0.395^k

Triphenylmethane dye decolorization assay

Fifteen laccase producers were selected for the determination of their ability to decolorize one of the triphenylmethane dye, bromophenol blue and the results of the quantitative were shown in the Table 2. Isolates were significantly different for the dye decolorization ability as measured by the absorbance values ($p < 0.05$). While both *Perenniporia tephropora* species and *Coriolopsis caperata* isolate decolorized more than 70% of the dye by the 8th day, *Vanderbylia fraxinea* and *Gymnopilus dilepis* isolates decolorized the dye by 70% after three weeks. Each treatment in triplicates showed the decolorization of the dye and no decolorization was observed in negative controls (Table 2). Qualitative test results were indicative of quantitative results. Other laccase positive fungal isolates displayed low decolorization efficiency (below 50%). Non-laccase producer, IMF5, had the least decolorization efficiency (below 10%). According to the Pearson's correlation analysis, there was a strong and significant correlation ($R = 0.64$, $P = 0.023$) between the dye decolorization and laccase production abilities as measured in absorbance values.

Discussion:

Here we reported the presence of 23 different fungal species present in total of 35 decaying hardwood samples (each with 1 cm²) and out of them, 11 species; *Coprinellus aureogranulatus*, *Coriolopsis caperata*, *Hypoxylon fragiforme*, *Neoscytalidium hyalinum*, *Perenniporia tephropora*, *Phanerochaete australis*, *Phialophora alba*, *Pleurostoma richardsiae*, *Paecilomyces formosus*, *Vanderbylia fraxinea* and *Xylogone sphaerospora*, were the first reports of Sri Lanka. This finding is important since it highlights that Sri Lankan fungal diversity studies are lagged behind though it is one of the 34 bio diversity hot spots in the world. Except a single study conducted by Ediriweera *et al.* [32] where 14 out of 49 fungal fruiting bodies were identified up to the species

level from ‘Sigiriya’ forest in the dry zone, our study is the first to use molecular level fungal species determination on decaying hardwoods in Sri Lanka. It is also important to note that two isolates, F3/TW5 and F12/DHF1 did not show sufficient sequence homology for any of the published records in the GenBank and tentatively identified as *Scytalidium* sp. (86%) and *Hexagonia* sp. (81%) and warrants further studies.

It was hypothesized that fungal species capable of degrading hardwoods are also high laccase producers. Presence of laccase has been determined by the formation of reddish brown color due to oxidation of guaiacol by laccase in many studies [33,34] and was implemented in the present study. Among the tested species, *Perenniporia tephropora* produced the highest amount of laccase and this finding is in line with Younes *et al.* [35]. There was no significant difference between *P. tephropora* and *Coriolopsis caperata* for the laccase production. Other best laccase producers were *Gymnopilus dilepis*, *Fusarium solani* and *Vanderbylia fraxinea*. Though the mother plant species of most of the decayed wood pieces were not known, it is interesting to note that *P. tephropora* and *V. fraxinea* were isolated from decaying woods of two economically and medicinally important hardwood species, Ebony (*Diospyros ebenum*) and Neem (*Azadirachta indica*) respectively. *Diospyros ebenum* also known as black wood is native to India and Sri Lanka and belongs to one of the hardest wood species resistant to withering and fungal infections. Neem is also known to contain numerous chemical compounds with medicinal importance and also known as the tree of the 21st century by the United Nations. Specially we tested whether these two fungi can utilize *Diospyros ebenum* wood dust as the sole C source and confirmed to be able to grow on wood agar palates (observational study only). Fungi isolated from the decaying woods of these plants were among the top laccase producers supporting our original hypothesis. Since Hori *et al.* (2017) found that white rot polyporales show greater enzymatic diversity than brown rot

polyporales [36] and both these fungal species were isolated from white rot hardwoods, it is worth conducting a full genomic analysis.

We found that the laccase production was positively correlated with triphenylmethane dye decolorization. Laccase and other ligninase enzyme extract's direct involvement in dye decolorization has been previously reported [37,38,39]. We also found that *P. tephropora* and *C. caperata* were the strongest bromophenol blue decolorizers similar to previous studies [35,40,41]. However, to the best of our knowledge triphenylmethane dye decolorization ability and laccase production of *Vanderbylia fraxinea* and *Gymnopilus dilepis* has never been reported in the fungal kingdom before and we report it for the first time.

These findings were of high importance, since it is known that synthetic dyes used in the textile, paper, leather and cosmetic industries are highly toxic, resistant to light, chemical and microbial degradation. Among many types of dyes, triphenylmethane dyes are resistant to enzymatic decolorization and it is time consuming [42]. Laccases require no H₂O₂ for oxidation reactions like other oxidases and that makes laccase an important enzyme in bio remediation. In the phylogenetic analysis, though laccase production is not monophylectic, all the high laccase producers were clustered together in a single clade of basidiomycetes. Though Ascomycetes have laccase producing ability, they produce relatively low amounts. It has also been reported that number of laccase gene copies could vary among species depending on the life history traits [43].

Finally, this study reported 11 wood decay fungal species in Sri Lanka as first reports highlighting the importance of a thorough fungal diversity study in Sri Lanka since there is a high potential to report novel biotechnologically important species. It was also proved that most of the decaying

wood associated fungi are indeed can utilize wood as the sole C source. To the best of our knowledge, laccase production and triphenylmethane dye decolorization ability of *Vanderbylia fraxinea* and *Gymnopilus dilepis* have never been reported in the fungal kingdom before. It was also found that laccase production was positively correlated with the dye decolorization ability. Therefore, the selected isolates have very high potential in applying for a greener biotechnology.

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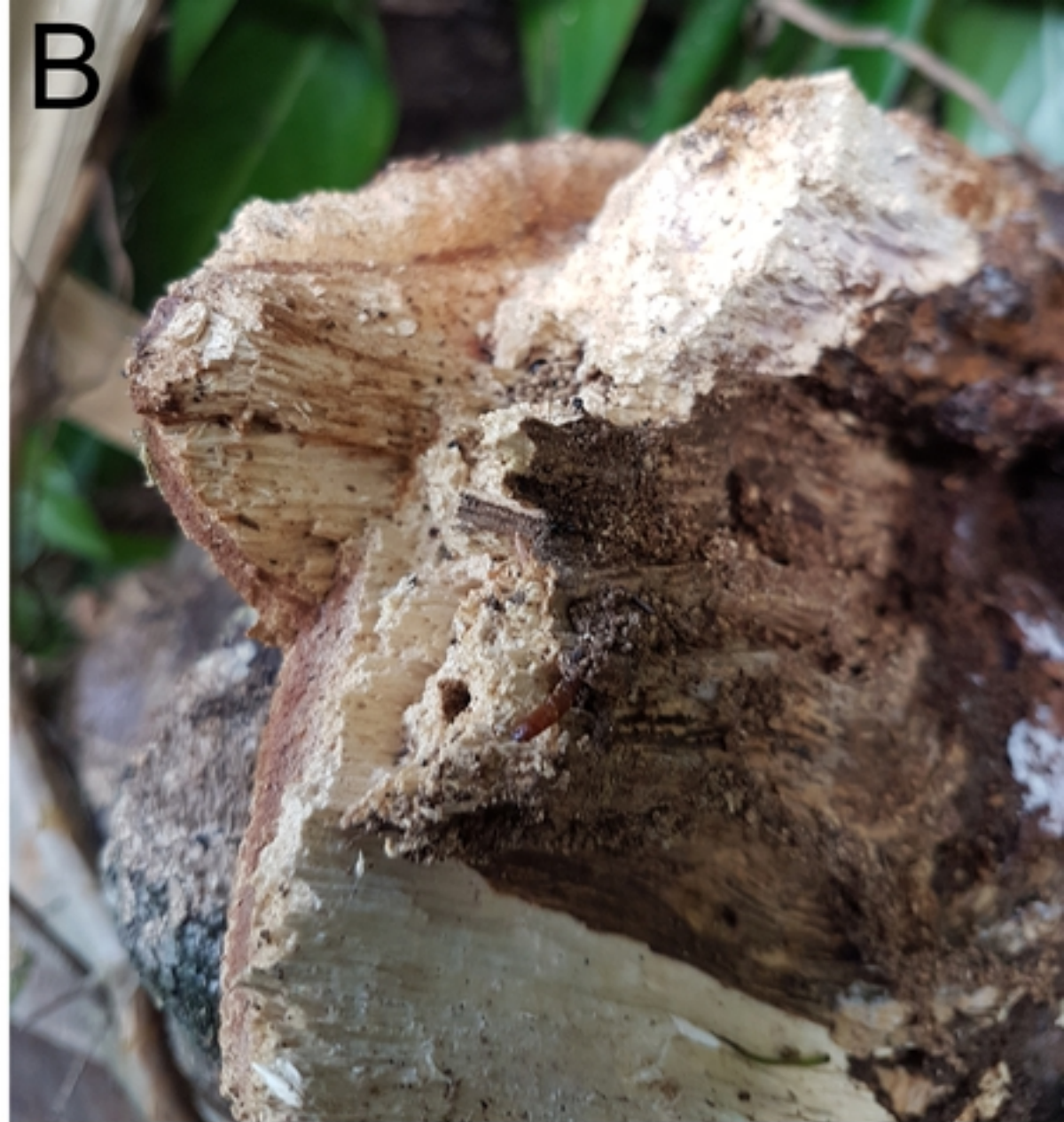


Fig 1

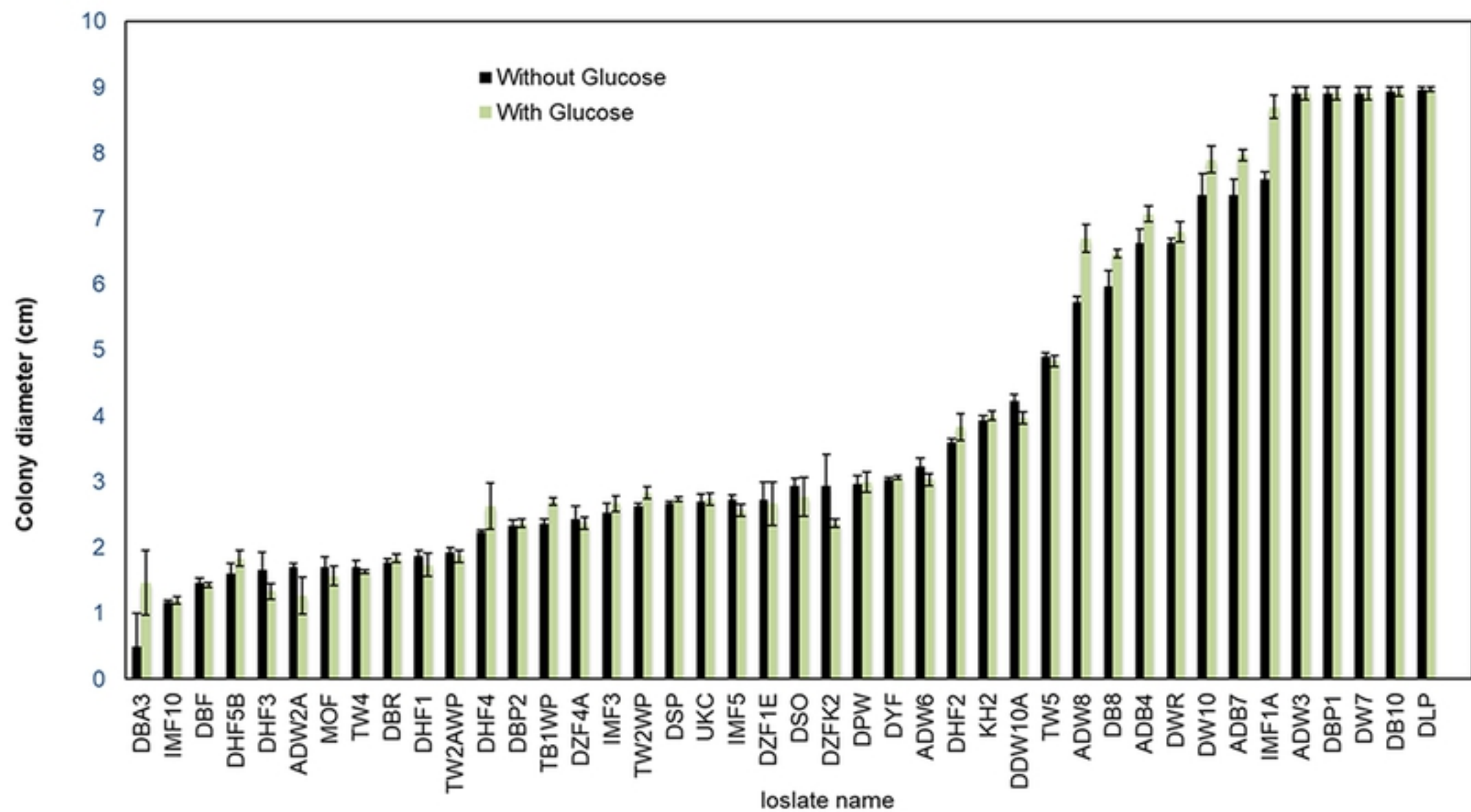


Fig 2

A

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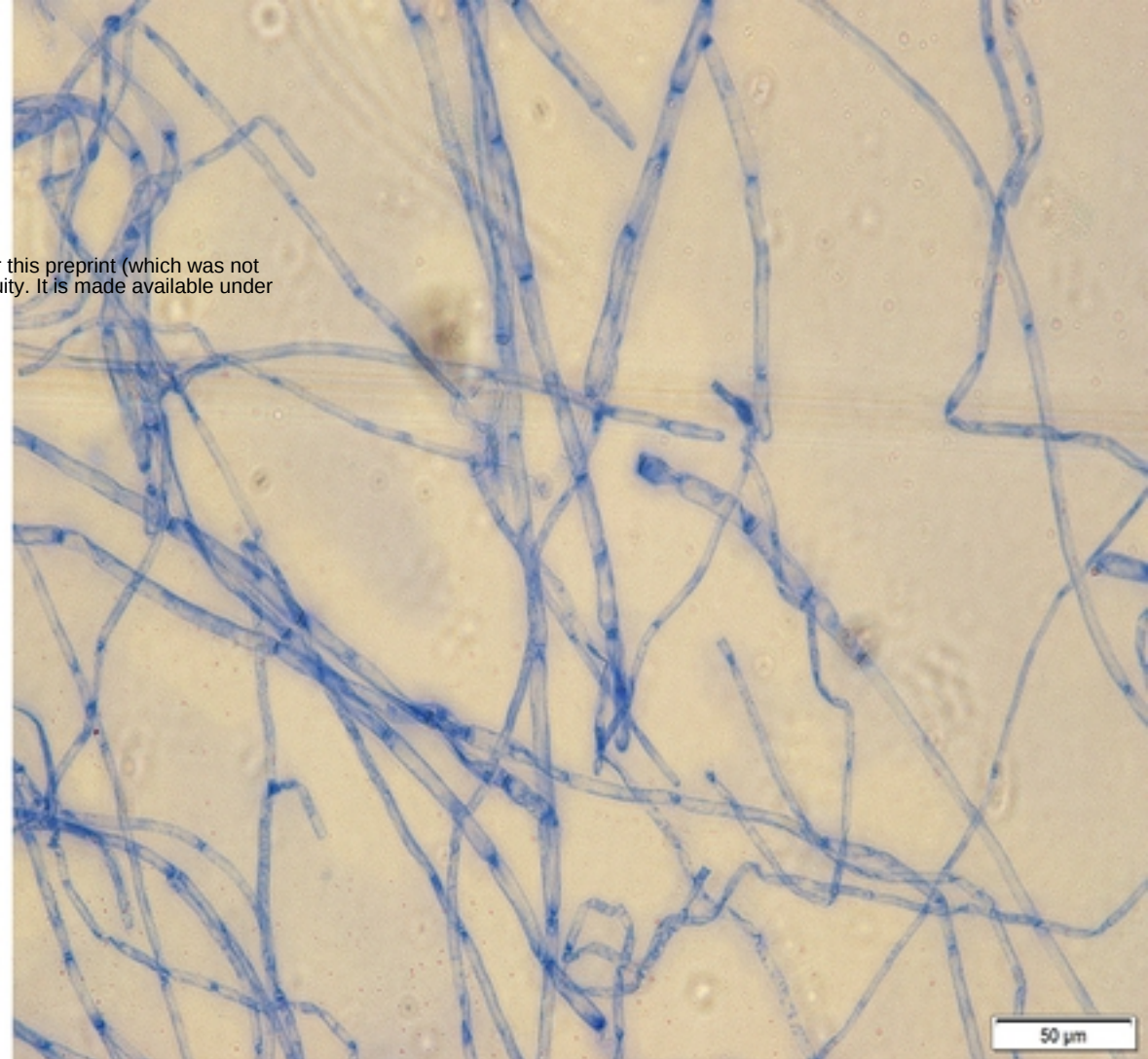
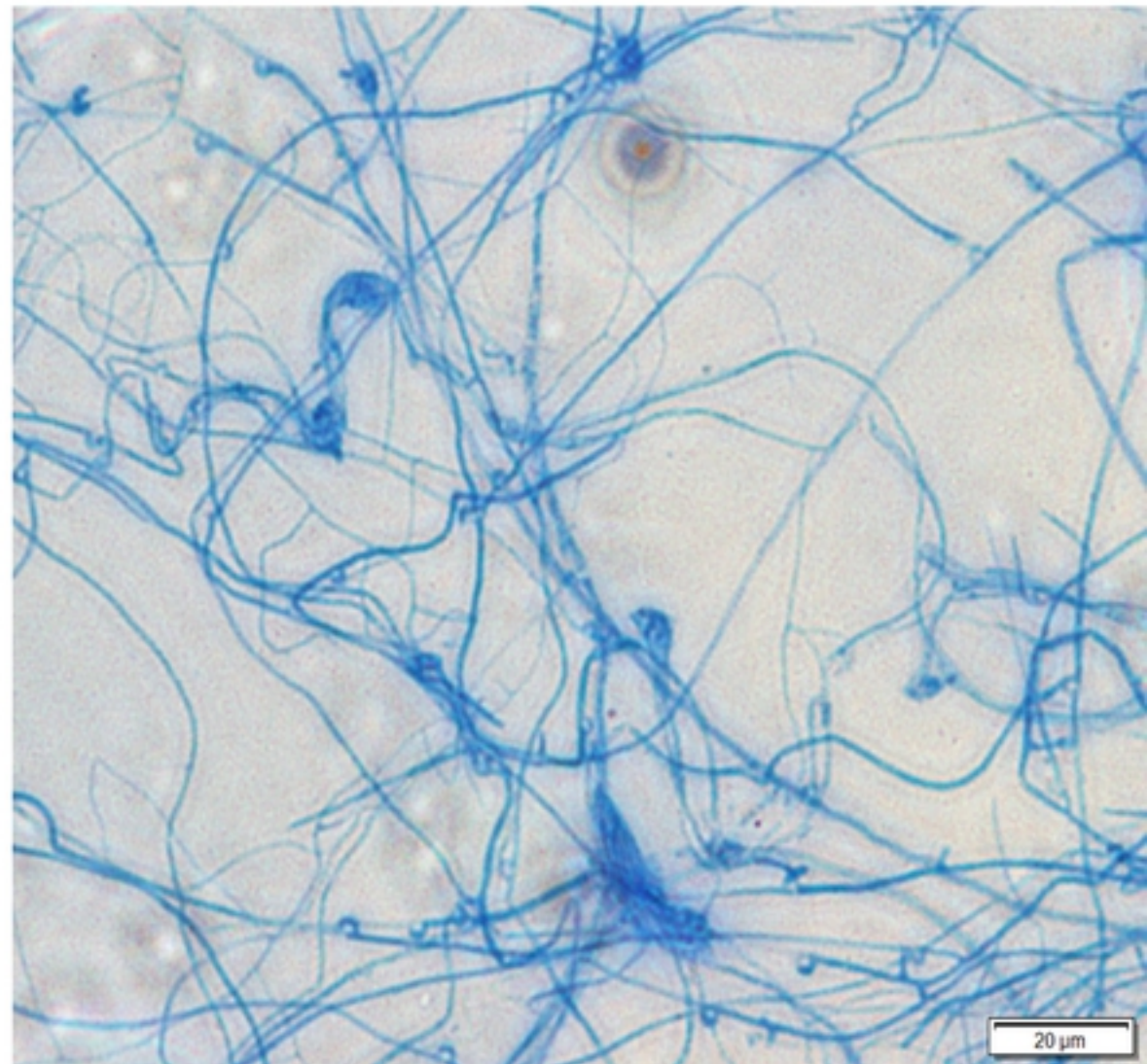
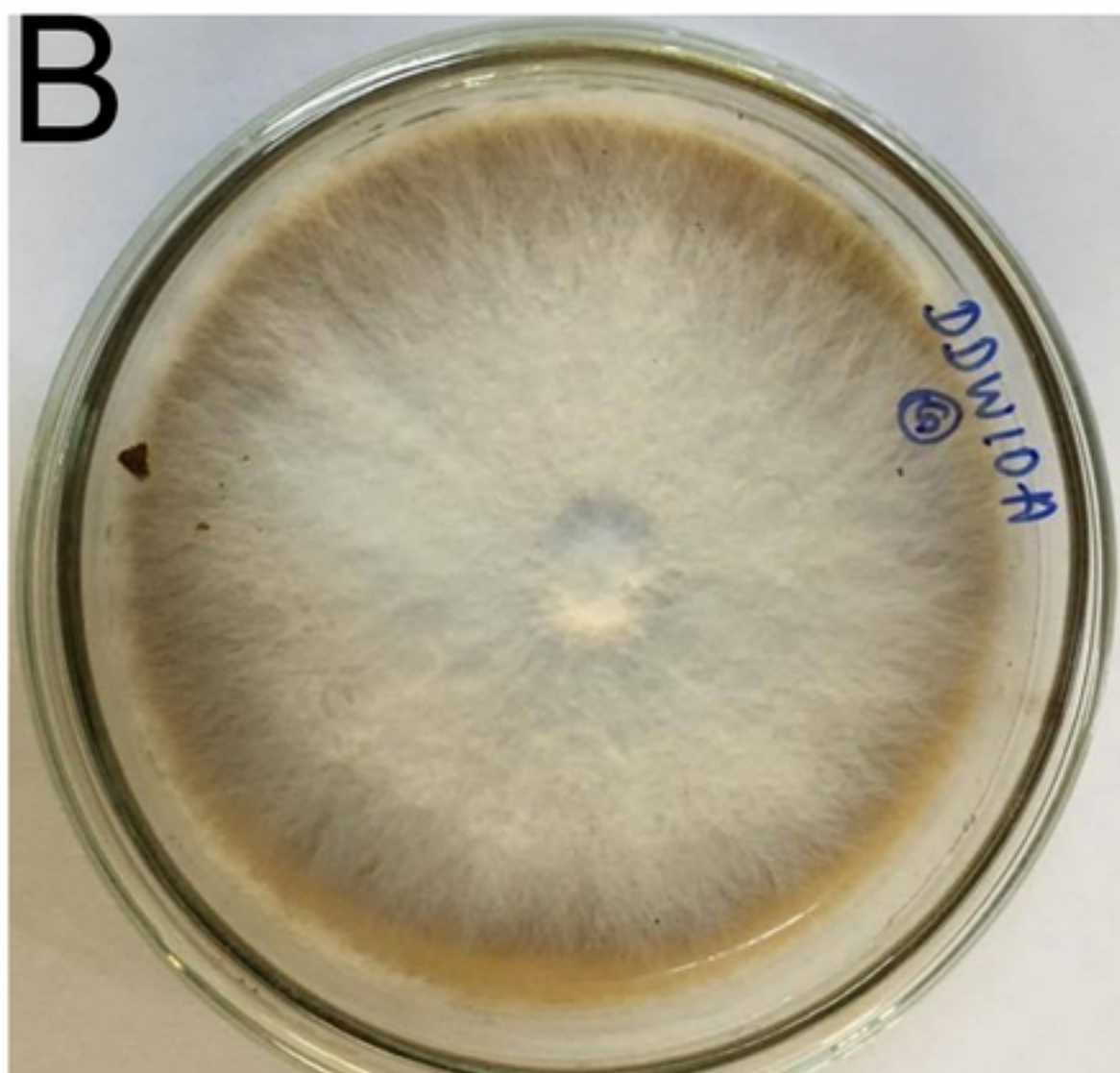
**B**

Fig 3

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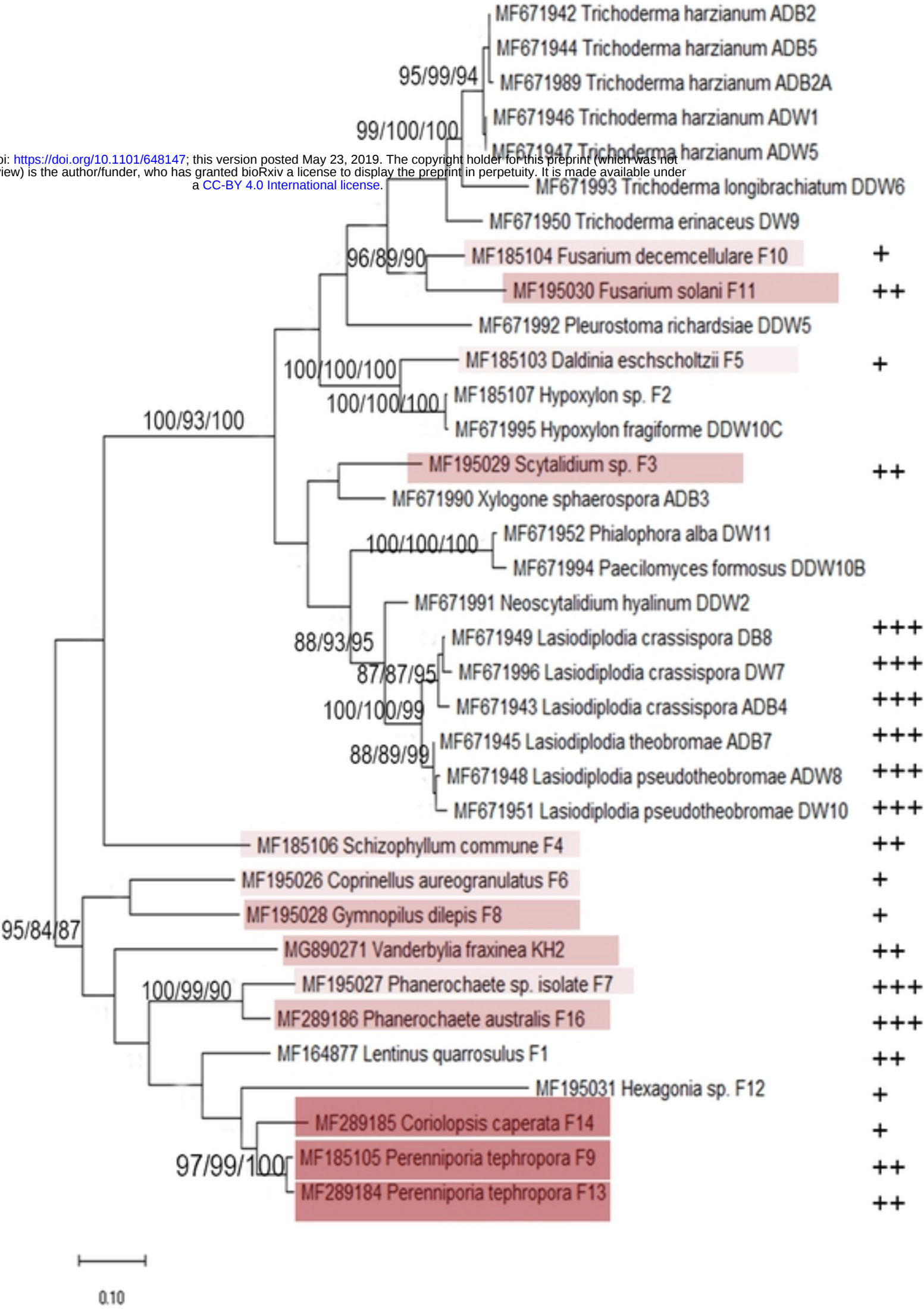


Fig 4