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

3

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15 MPSMD: Conducted wet lab work and manuscript writing

16 ASD: Conducted wet lab work and manuscript writing

17 PW: Design experiments and manuscript writing

18 RNA: Conceptualized the idea, secured finding, design experiments and manuscript writing.

19

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20 # Abstract

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

40

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

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42 # Introduction

43

44 Fungi are considered to be miniature metabolic factories having versatile secondary metabolites
45 and enzymes with the potential to be used as work horses in diverse array of biotechnological
46 applications [1]. Based on the Hawksworth's [2] estimation, there are abut of 1.5 million fungal
47 species present on earth, which is considered as the baseline estimation. However, recent
48 metagenomics studies suggested that actual numbers might be closer to 3.5 to 5.1 million species
49 or much higher than this [3]. This uncertainty in the numbers is partially due to lack of advanced
50 molecular based thorough studies in the tropics where incredibly rich diversity has been reported
51 [4]. Hawksworth [5] also suggested that much of the undescribed fungal species could be present
52 in the tropics and it is reviewed in Aime and Brearley [4].

53 Sri Lanka, a tropical island in the Indian ocean along with the Western Ghats, belongs to one of
54 the 34 biodiversity hotspots in the world. Though its plant and animal diversity is well studied
55 [6,7], microbial studies, especially fungal studies remain in its infancy. Furthermore, biodiversity
56 hotspot concept of Sri Lanka should not be an exception for microbial diversity including fungi.
57 However, most of the fungal studies in Sri Lanka have mainly focused on macro-fungi using
58 morphological characters [8]. Moreover, these studies have mainly concentrated on the wet zone
59 forests. On the other hand, dry zone forest ecosystems in the country spread over 22 % of area in
60 Sri Lanka, whereas the total forest cover is about 26.6%. Dimbulagala (7°51'40.5"N
61 81°07'05.5"E) is an isolated hill covered with dry zone forest and it is rich with strong and
62 economically important hardwood bearing plant species. This region is contacted with minimum
63 anthropological activities for many years mainly due to 30-year long civil war in this region and
64 was the study site of the current study. An unexplored niche, decaying hardwood, of

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

67 For a healthy forest ecosystem, it is critical to recycle carbon stored in these hardwood and litter.
68 Among many organisms such as beetles, flies, slime molds, bacteria, slugs and snails, primary
69 decomposers that recycle carbon are fungi. It is known that wood, specially hardwood is a
70 challenging substrate to degrade as it has very low nitrogen content (commonly C: N is about
71 500:1) and the presence of various fungal toxic compounds. Therefore, fungi specially
72 basidiomycetes have evolved to degrade the hardwood structure consisted of cellulose,
73 hemicellulose and the strongest natural polymer, lignin. They secrete major lignin modifying
74 enzyme families such as lignin peroxidase, manganese peroxidase, versatile peroxidase and
75 laccase [9]. Among them, fungal laccases have attracted the attention of scientific community due
76 to its high redox potential and versatile catalytic properties compared to the laccases from other
77 sources such as plants and bacteria [10]. Sri Lankan dry zone forests, including Dimbulagala
78 forest reserve are rich in hardwood bearing plant species such as *Diospyros ebenum*, *Manilkara*
79 *hexondra*, *Vitex pinnata, Diospyros chaetocarpa*, *Terminalia bellirica, Chloroxylon swietenia* and
80 *Berrya cordifolia* [11–13]. These plant species produce hardwood with high economic value and
81 are resistant to degradation. However, mycelial mats and mushrooms were often observed in
82 decaying hardwoods in these areas indicating the involvement of fungal for the decay process. If
83 that is the case, fungal species found in association with these decaying hardwood will be able to
84 utilize wood as the sole carbon source. Therefore, the second objective was to assess the ability of
85 the fungal isolates to grow on wood dust media. It was hypothesized that fungal species capable
86 of growing on such hardwood have the ability to produce high amounts of laccases.

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

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

99 Materials and Methods:

100 Sample collection

101

102 Sampling site was Dimbulagala historical forest reserve in Polonnaruwa district, Sri Lanka.
103 Research permits were obtained from the authorities and samples were collected and handled
104 ethically without harming living trees. Thirty-five decayed hard wood pieces of 5-6 cm length
105 showing brown and white rot symptoms were collected randomly along the trail to the top of the
106 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
107 zip lock plastic bags and transported to the laboratory at University of Kelaniya (Fig. 1). Host of
108 the decaying wood was recorded whenever possible. However, in most of the cases it was not

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109 possible to clearly identify the plant species that decayed wood pieces belonged to. Samples were
110 air dried for two days and stored in a refrigerator until further use.
111

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

114 Isolation of fungi into culture media

115

116 Decaying wood pieces (approximately 1 × 1 cm) were surface sterilized with 5% Clorox for 2
117 minutes followed by three serial washings with sterilized distilled water. Washed materials were
118 blot dried using sterilized filter papers. Samples were trimmed from the edges and cut into
119 approximately four pieces, placed on semi selective PDA media supplemented with streptomycin
120 (100 µg/mL). Another isolation step was carried on semi selective medium slightly modified from
121 Baumgartener et al. [19] by supplementing PDA with streptomycin (100 µg/mL) and fungicide
122 carbendazim (4 µg/mL) to inhibit fast growing ascomycetes. Plates were incubated at room
123 temperature for one week. All the fungal colonies originated from the wood pieces were sub
124 cultured and pure cultures were obtained by hyphal tip isolation method. Pure cultures of 55 fungal
125 isolates were obtained and vouchered both in sterile water and at -20 ℃ in Whatman number 1
126 filter papers at the Department of Botany, University of Kelaniya, Sri Lanka and assigned strain
127 IDs.

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

129 Ability of isolated fungi to utilize wood as the sole carbon (C) source was tested according to Swe
130 [20]. During this assay most of the isolates those with morphological characters similar to

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131 *Trichoderma* spp. were excluded and therefore, out of 55, 43 isolates were used in further studies.
132 First, saw dust of *Mangifera indica* was passed through 2 mm sieve. Wood agar plates were
133 prepared with saw dust (2% w/v), stock salt solution (5 % v/v), Hutner's trace elements (0.02 %
134 v/v), with and without supplementing glucose (0.05% w/v) and agar (1.5 % w/v) followed by Swe
135 [20]. Both wood agar plates with and without glucose were inoculated with 5 mm-diameter
136 mycelial disc obtained from actively growing edge of 7-day old culture from each isolate. Plates
137 were incubated at the room temperature (28 ± 2 ºC) for three days. Colony diameters were
138 measured in two-dimension perpendicular to each other. Experiment was repeated once.

139

140 Morphological and molecular identification of fungi

141

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

146 Out of all the isolates, 35 isolates that showed variable morphological features indicating
147 putatively different species were selected for molecular level identification. Three mycelial plugs
148 from actively growing colonies were inoculated into 50 mL Potato Dextrose Broth (PDB)
149 supplemented with streptomycin (100 µg/mL). Flasks were incubated in a rotary shaker (Stuart-
150 SSL1, UK) at 120 rpm at room temperature for 5-7 days. Mycelia were separated, washed with
151 500 µL of sterilized distilled water and squeeze dried with sterilized filter papers. DNA was
152 extracted following modified method of Cenis [21]. DNA Pellet was re-suspended in TE buffer
153 (50-100 µL) and stored at -20°C until further analysis. Quality of extracted DNA was visually

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154 observed on 0.8% agarose containing 0.5 µg/ml of Ethidium bromide. Polymerase Chain Reaction
155 (PCR) was performed with universal ITS1 and 4 or 4 and 5 primer pairs [22]. Reaction mixture
156 consisted of 1x Colorless GoTaq® Flexi Buffer, 2 mM $MgCl_2$, 200 µM each dNTP, 0.5 µM
157 forward and reverse primers, 1.25U of GoTaq® DNA Polymerase (Promega Inc., USA) and <0.5
158 µg template DNA. PCR reaction was performed in a thermal cycler (Veriti® 96-Well Thermal
159 Cycler, ABI Biosystems, USA) as published in Maduranga et al.[23]. Nuclease free water was
160 added as the negative control and PCR products were separated using agarose gel electrophoresis
161 and visualized under a Gel Documentation System (Quantum ST5, Germany). Pure PCR products
162 were sequenced following Sanger dideoxy chain termination technology at the Genetech Institute,
163 Colombo, Sri Lanka. Sequences were manually edited using BioEdit sequence Alignment Editor
164 (Version7.2.5)[24]. Using Basic Local Alignment Search Tool (BLASTn), nucleotide sequences
165 were compared with the authentic sequences available in the National Center for Biotechnology
166 Information (NCBI) database and species were identified. All sequences were deposited in the
167 GenBank and the accession numbers obtained are shown in Table 1.

168 Molecular phylogenetic analysis

169 DNA sequences were aligned using a multiple sequence alignment algorithm, Multiple Sequence
170 Comparison by Log- Expectation (MUSCLE) [25], implemented in MEGA ver. X [26]. Gblocks
171 0.91b was used to eliminate poorly aligned positions and divergent regions of the alignment [27].
172 All characters were equally weighted and the gaps were treated as missing data. No out group was
173 used and tree was unrooted. The evolutionary history was inferred using several phylogenetic tree
174 construction methods, Maximum Likelihood, maximum parsimony and neighbor-joining,
175 implemented in MEGA ver. X software. For Maximum Likelihood method, initial tree(s) for the
176 heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to

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

181 Qualitative determination of laccase production by fungal spp.

182

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

189 Quantitative determination of laccase production by fungal spp.

190

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

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200 (Stuart-SSL1, UK) (120 rpm) and 1.00 mL of each sample was collected once in the 5th, 8th and
201 12th day during the incubation period. The collected samples were centrifuged at 5100 rpm for 15
202 min and the supernatant (here after referred as enzyme extract) was stored at -20℃ [20]. Reaction
203 mixture (5 mL) contained 3.90 mL acetate buffer (10 mM, pH 5.0), 1.00 mL guaiacol (1.76 mM)
204 and 0.1 mL of the enzyme extract, and incubated at 25°C for 2 hrs. followed by the measuring of
205 absorbance at 450 nm (Thermo scientific Multiskan GO, Finland). In the blank, enzyme extract
206 was substituted with the buffer. Enzyme activity is considered to be corresponding with the
207 increase in absorbance at 450 nm as indicated in Arora and Sandhu [28]. One-way Analysis of
208 Variance (ANOVA) was carried out using Minitab 17 software (Minitab Inc., USA) to determine
209 if there is a significant difference among isolates for the laccase production. Experiment was
210 repeated once.

211 Triphenylmethane dye decolorization capacity of laccase producing 212 fungi

213

214 Conical flasks containing 25 mL of sterilized medium containing glucose (10 g/L), KH_2PO_4 (1
215 g/L), $MgSO_4$ (0.5 g/L), $CaCl_2$ (0.14 g/L), yeast extract (1 g/L), thiamine (0.0025 g/L) and
216 bromophenol blue (0.05%) were inoculated with 3 mycelial discs (5.00 mm) of 7-day old actively
217 growing colonies [29]. Flasks were incubated at room temperature for 21 days in the shaker
218 (Stuart-SSL1, UK) at 210 rpm. Two milliliters from each flask was obtained in 8th and 21st days
219 after incubation and centrifuged at 12,000 rpm for 5 minutes to obtain cell free supernatant.
220 Absorbance of each sample was measured at 590 nm (Thermo Scientific Orion aquamate 8000
221 UV-VIS spectrophotometer, USA) following Nidadavolu *et al.* [30]. Previously used IMF5 was
222 used as the negative control. In addition, media with plain PDA plugs was also served as a negative
223 control. The decolorization efficiency was determined using the following equation [31],

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224

$$\% \, decolorization = \left[\frac{A \, initial - A \, final}{A \, initial}\right] * 100\% \quad (225)$$

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

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244 (6.0-3.0 cm) and slow growers (<3.0 cm). Figure 2 shows the variation in colony diameters on
245 both media for the 43 isolates. Raw data were deposited in Dryad repository
246 https://doi.org/10.5061/dryad.g7f4m0j

247

248

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

251

252 Identification of fungi

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

255

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

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

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264

265

266

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

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

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

269

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**

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

290

291 Qualitative determination of laccase production by fungal spp.

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

296 Quantitative determination of laccase production by fungal spp.

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

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

Isolate	Species name	Laccase production Mean absorbance values ± SD			Percentage decolorization of bromophenol blue Mean % decolorization ± SD	
		5th day	8th day	12th day	8th day	21st day
DDW10A	*Perenniporia tephropora*	0.158 ± 0.004[a]	0.168 ± 0.006[a]	0.166 ± 0.003[a]	71.173 ± 0.974[a]	89.29 ± 0.680[a]
DHF2	*Perenniporia tephropora*	0.153 ± 0.006[a]	0.152 ± 0.008[a]	0.142 ± 0.009[b]	70.800 ± 0.230[a]	87.19 ± 0.506[b]
ADW2A	*Coriolopsis caperata*	0.144 ± 0.006[a,b]	0.157 ± 0.004[a]	0.115 ± 0.008[c]	70.087 ± 0.172[a]	84.63 ± 0.569[c]
TW2AWP	*Gymnopilus dilepis*	0.128 ± 0.009[b,c]	0.124 ± 0.004[b]	0.131 ± 0.006[b,c]	54.753 ± 0.396[b]	74.18 ± 0.742[e]
DZFK2	*Fusarium solani*	0.122 ± 0.002[c]	0.122 ± 0.005[b]	0.121 ± 0.002[c]	14.923 ± 1.236[f]	15.11 ± 0.196[i]
KH2	*Vanderbylia fraxinea*	0.117 ± 0.005[c]	0.124 ± 0.001[b]	0.139 ± 0.004[b]	52.047 ± 0.365[c]	79.07 ± 0.396[d]
TW5	*Scytalidium* sp.	0.111 ± 0.010[c,d]	0.133 ± 0.006[b]	0.119 ± 0.008[c]	11.427 ± 0.396[g]	18.45 ± 0.879[h]
DLP*	n/a	0.098 ± 0.006[d,e]	0.085 ± 0.003[c]	0.088 ± 0.003[d]	39.683 ± 0.455[d]	40.17 ± 0.172[f]
ADW3	*Phanerochaete australis*	0.083 ± 0.007[e,f]	0.119 ± 0.008[b]	0.115 ± 0.005[c]	11.463 ± 0.800[g]	24.61 ± 0.287[g]
DHF5B	*Fusarium decemcellulare*	0.081 ± 0.002[f]	0.079 ± 0.007[c]	0.066 ± 0.005[e]	n/a	n/a
DPW	*Schizophyllum commune*	0.069 ± 0.003[f]	0.074 ± 0.001[c,d]	0.089 ± 0.004[d]	12.627 ± 0.597[g]	12.03 ± 0.919[j]
DZF4A	*Coprinellus aureogranulatus*	0.050 ± 0.004[g]	0.059 ± 0.006[d,e]	0.086 ± 0.006[d]	17.437 ± 0.469[e]	18.53 ± 0.168[h]
IMF1A	*Phanerochaete* sp.	0.047 ± 0.005[g]	0.059 ± 0.007[d,e]	0.039 ± 0.004[f]	n/a	n/a
DBF	*Daldinia eschscholtzii*	0.045 ± 0.007[g]	0.046 ± 0.003[e]	0.044 ± 0.001[f]	n/a	n/a

318 **were significantly different from each other ($p<0.05$).**
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322 * repeated attempts of DNA barcoding was unsuccessful n/a- not determined
323

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IMF10*	n/a	0.040 ± 0.002[g]	0.116 ± 0.003[c]	0.112 ± 0.005[d]	13.227 ± 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]

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325 # Triphenylmethane dye decolorization assay

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

338 # Discussion:

339 Here we reported the presence of 23 different fungal species present in total of 35 decaying
340 hardwood samples (each with 1 cm^2) and out of them, 11 species; *Coprinellus aureogranulatus,*
341 *Coriolopsis caperata, Hypoxylon fragiforme, Neoscytalidium hyalinum, Perenniporia tephropora,*
342 *Phanerochaete australis, Phialophora alba, Pleurostoma richardsiae, Paecilomyces formosus,*
343 *Vanderbylia fraxinea* and *Xylogone sphaerospora,* were the first reports of Sri Lanka. This finding
344 is important since it highlights that Sri Lankan fungal diversity studies are lagged behind though
345 it is one of the 34 bio diversity hot spots in the world. Except a single study conducted by
346 Ediriweera *et al.* [32]where 14 out of 49 fungal fruiting bodies were identified up to the species

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

352 It was hypothesized that fungal species capable of degrading hardwoods are also high laccase
353 produces. Presence of laccase has been determined by the formation of reddish brown color due
354 to oxidation of guaiacol by laccase in many studies [33,34] and was implemented in the present
355 study. Among the tested species, *Perenniporia tephropora* produced the highest amount of laccase
356 and this finding is in line with Younes *et al.* [35]. There was no significant difference between *P.*
357 *tephropora* and *Coriolopsis caperata* for the laccase production. Other best laccase producers were
358 *Gymnopilus dilepis, Fusarium solani* and *Vanderbylia fraxinea.* Though the mother plant species
359 of most of the decayed wood pieces were not known, it is interesting to note that *P. tephropora*
360 and *V. fraxinea* were isolated from decaying woods of two economically and medicinally
361 important hardwood species, Ebony (*Diospyros ebenum*) and Neem (*Azadirachta indica*)
362 respectively. *Diospyros ebenum* also known as black wood is native to India and Sri Lanka and
363 belongs to one of the hardest wood species resistant to withering and fungal infections. Neem is
364 also known to contain numerous chemical compounds with medicinal importance and also known
365 as the tree of the 21st century by the United Nations. Specially we tested whether these two fungi
366 can utilize *Diospyros ebenum* wood dust as the sole C source and confirmed to be able to grow on
367 wood agar palates (observational study only). Fungi isolated from the decaying woods of these
368 plants were among the top laccase producers supporting our original hypothesis. Since Hori et al.
369 (2017) found that white rot polyporales show greater enzymatic diversity than brown rot

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

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

379 These findings were of high importance, since it is known that synthetic dyes used in the textile,
380 paper, leather and cosmetic industries are highly toxic, resistant to light, chemical and microbial
381 degradation. Among many types of dyes, triphenylmethane dyes are resistant to enzymatic
382 decolorization and it is time consuming [42]. Laccases require no H_2O_2 for oxidation reactions
383 like other oxidases and that makes laccase an important enzyme in bio remediation. In the
384 phylogenetic analysis, though laccase production is not monophylectic, all the high laccase
385 producers were clustered together in a single clade of basidiomycetes. Though Ascomycetes have
386 laccase producing ability, they produce relatively low amounts. It has also been reported that
387 number of laccase gene copies could vary among species depending on the life history traits [43].

388

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

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

397 # Acknowledgements

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A

B

Fig 1

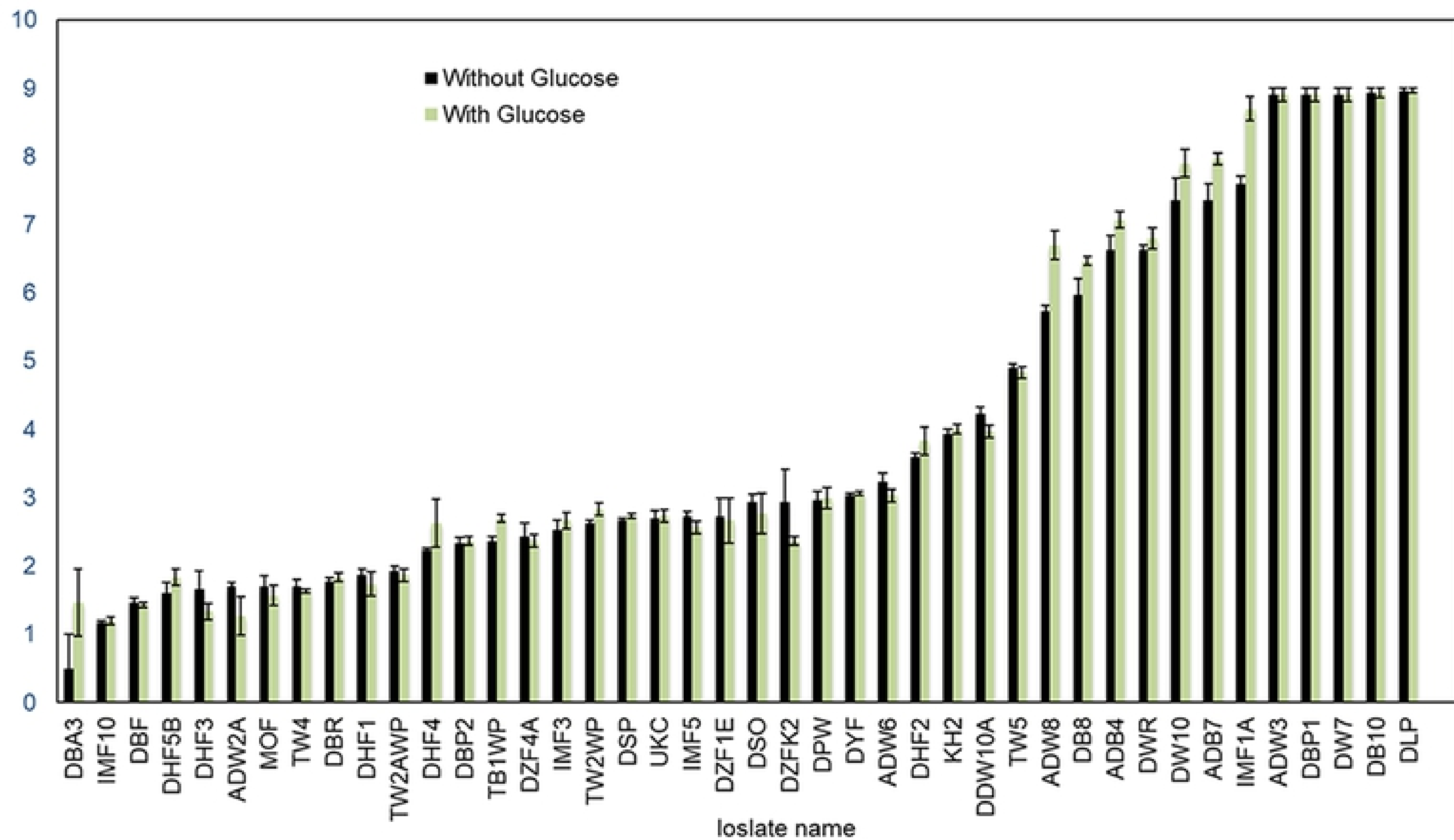

Fig 2

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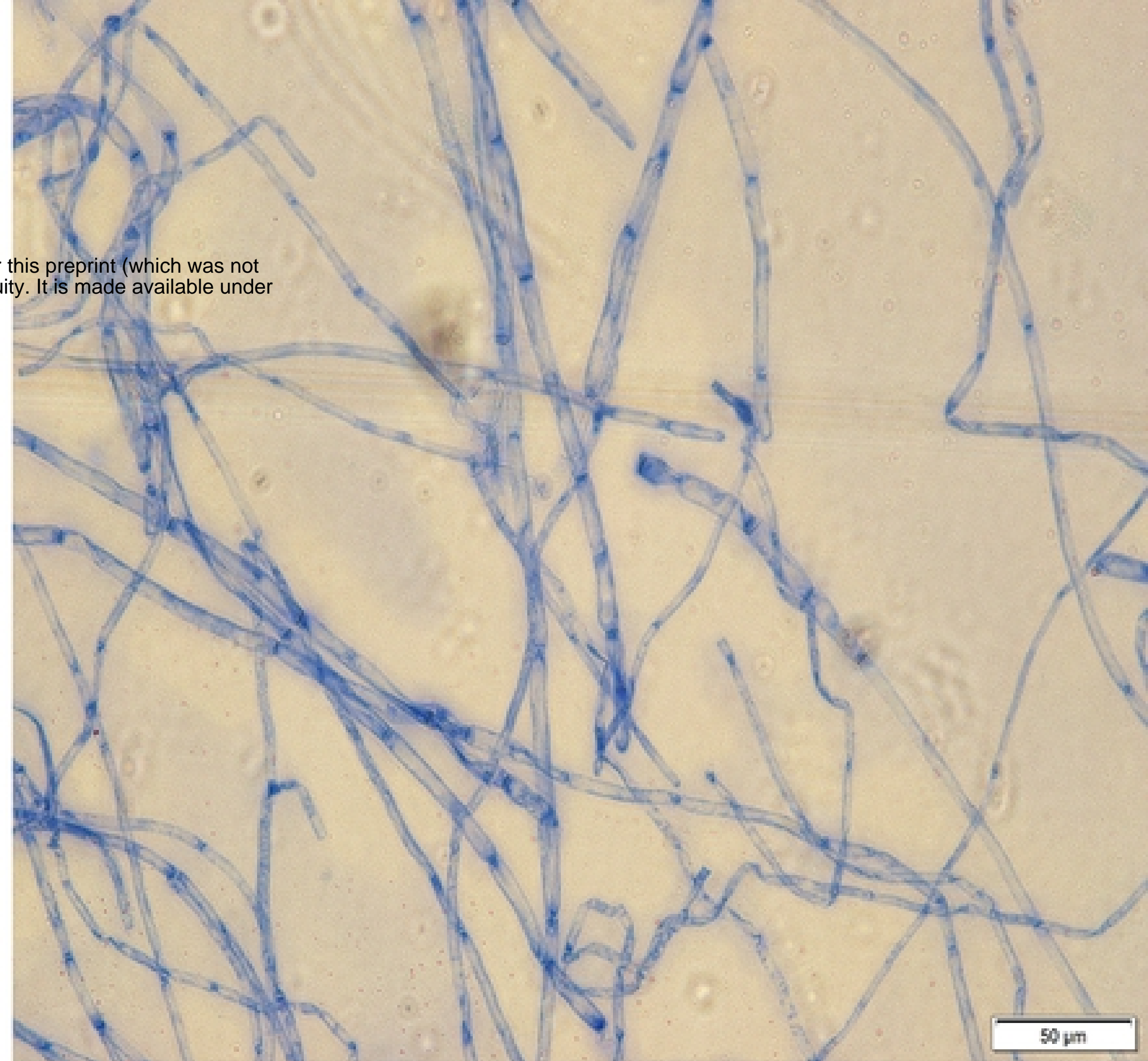

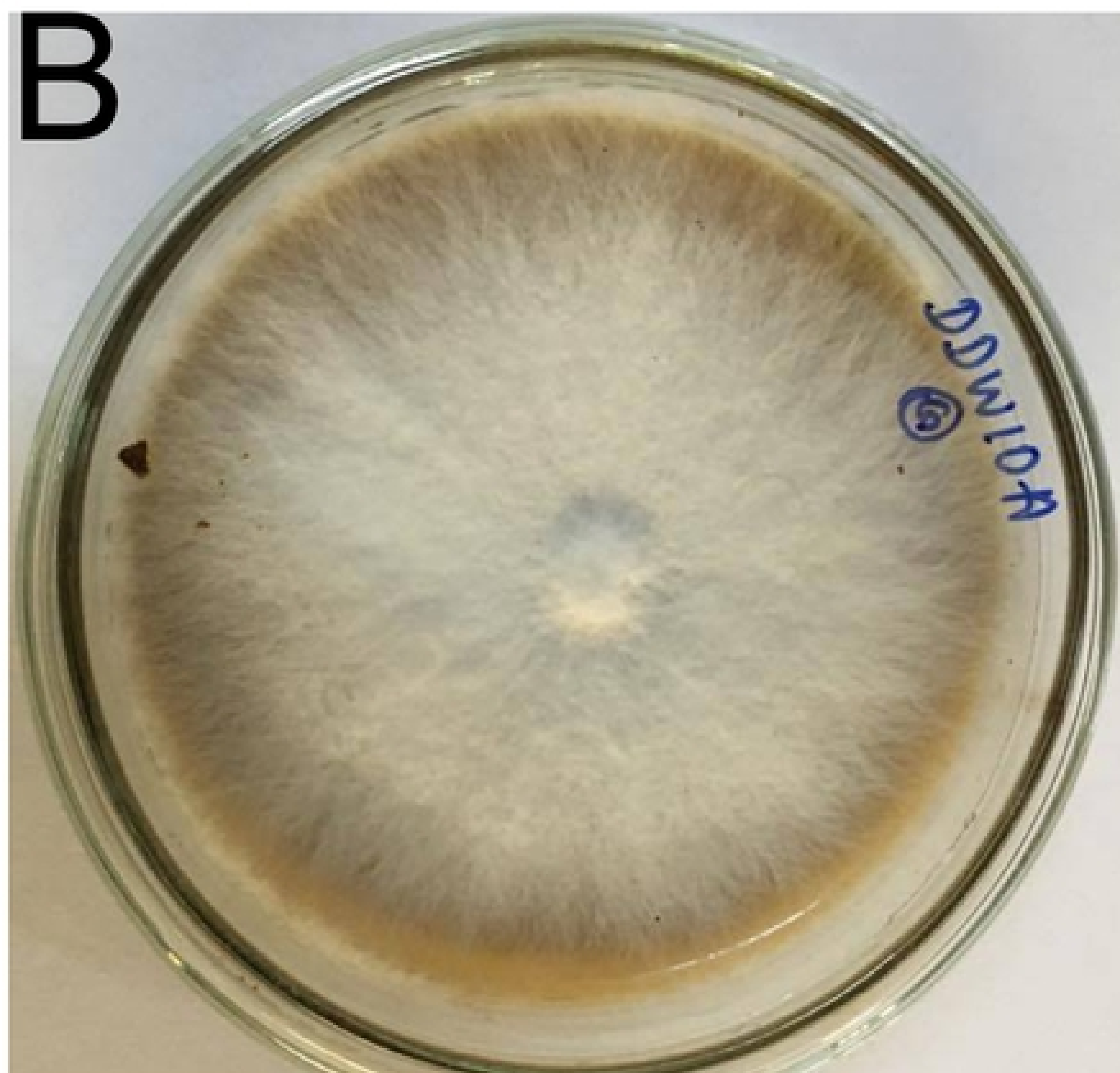

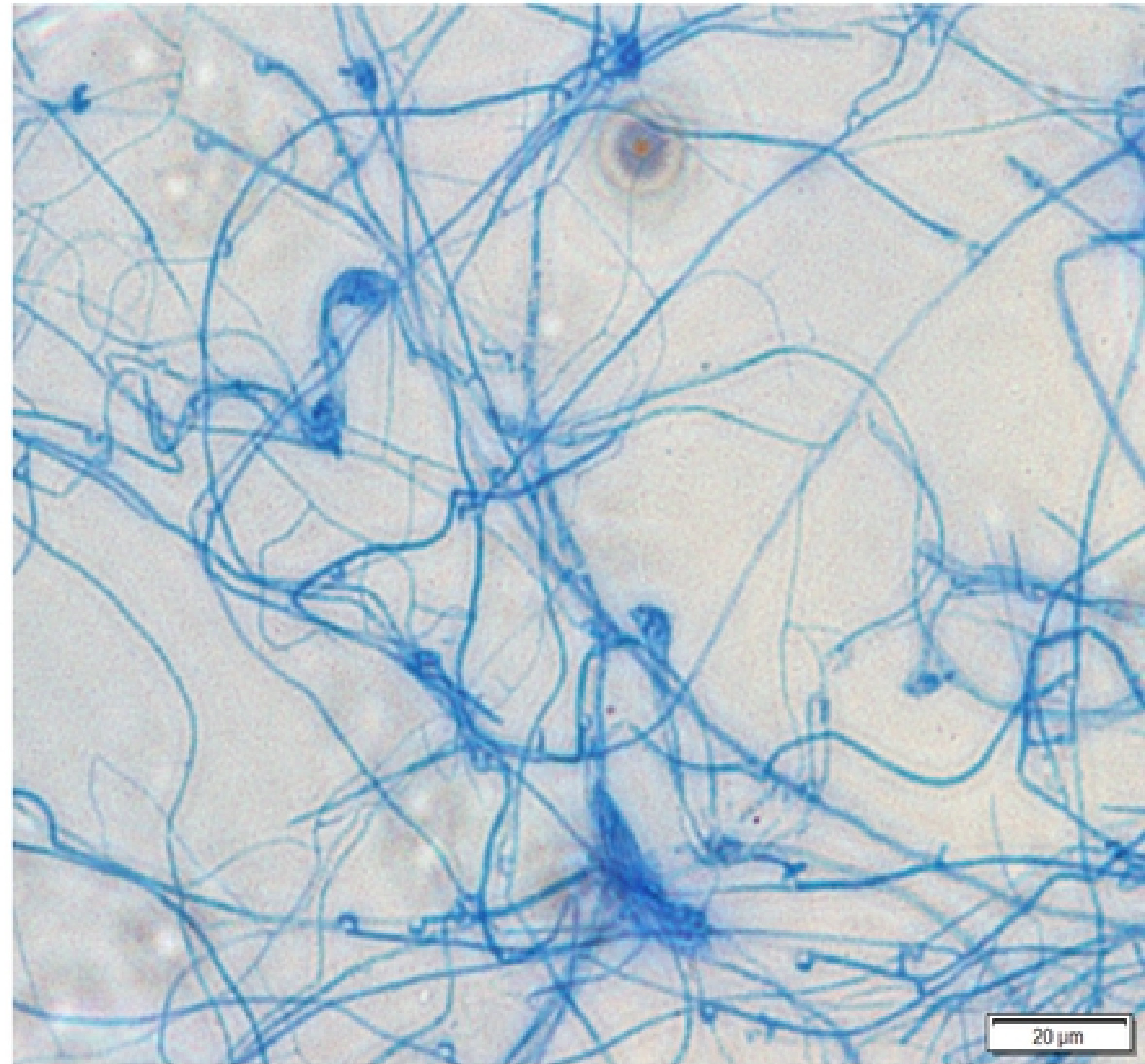

Fig 3

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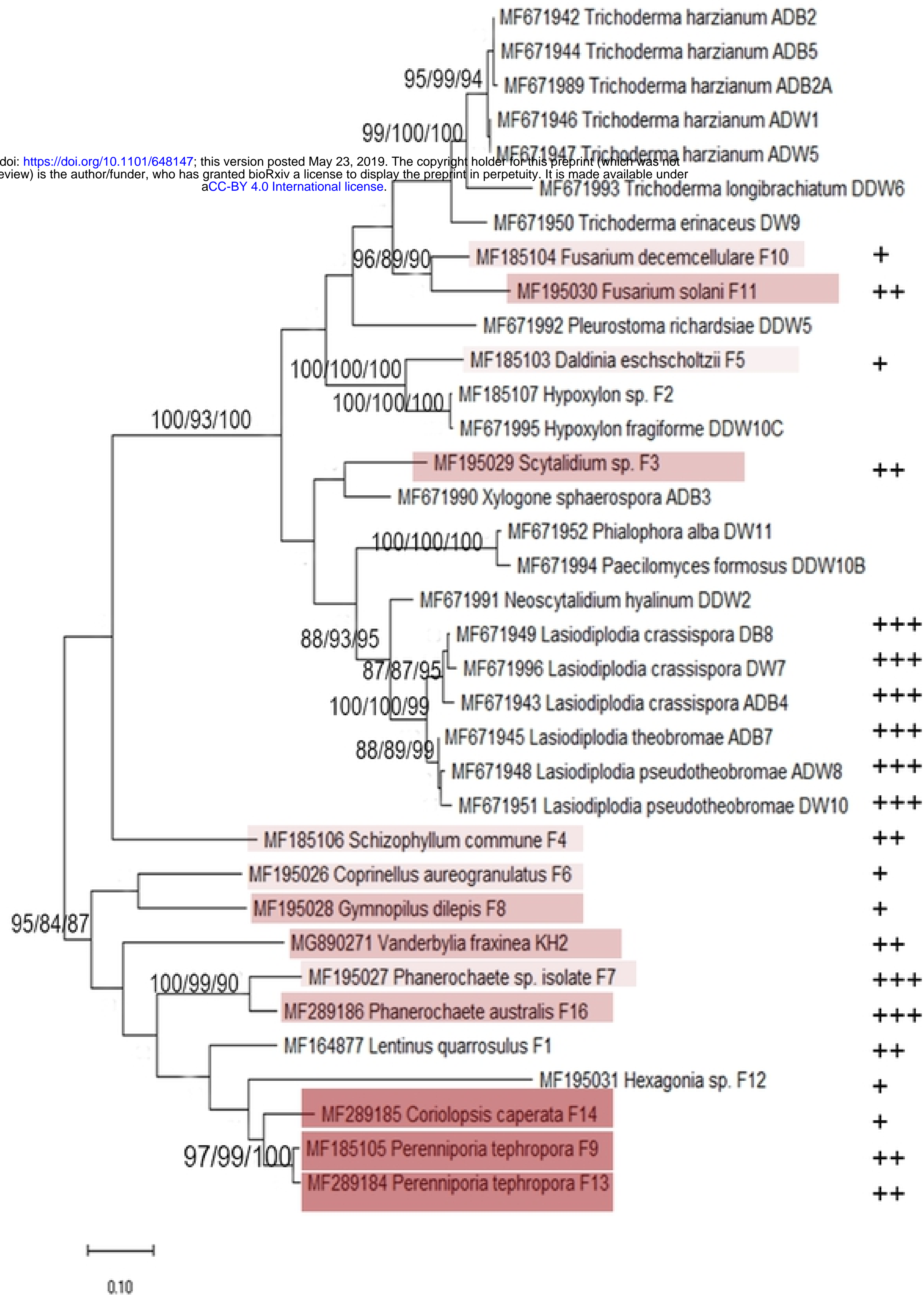

Fig 4