Authors' contributions
IM performed experiments and wrote the article; MAS helped in collection and isolation; GVM arranged the resources and conceived the idea; MSVG helped in data analysis and arranged the resources

ORIGINAL RESEARCH PAPER

Isolation and molecular identification of laccase-producing saprophytic/phytopathogenic mushroom-forming fungi from various ecosystems in Michoacán State, Mexico

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Abstract

The aim of this study was isolation and molecular identification of laccase-producing saprophytic/phytopathogen Basidiomycetes species from different geographic regions with dominant vegetation of *Pinus*, *Abies*, and *Quercus* spp. in the state of Michoacán, Mexico. Soil samples and visible mycelial aggregates were collected for fungal isolations. Soil samples were processed using a soil particle washing technique, where a selective Ascomycetes inhibitor and guaiacol, as an indicator of saprophytic Basidiomycetes growth, were used. Most of the isolates were obtained from samples collected in Parque Nacional, José Ma. Morelos (Km 23), Charo, Michoacán, Mexico. Based on sequence comparisons and phylogenetic analysis of internal transcribed spacer regions (ITS1-5.8S-ITS4) with respect to reference taxa, identification of saprophytic/phytopathogen Basidiomycetes species was carried out. In total, 15 isolates from 12 genera (i.e., *Bjerkandera*, *Coriolopsis*, *Ganoderma*, *Hexagonia*, *Irpex*, *Limonomyces*, *Psathyrella*, *Peniophora*, *Phlebia*, *Phlebiopsis*, *Trametes*, and *Trichaptum*) and one species from family Corticiaceae were identified. This study will be useful for further investigations on biodiversity of soil Basidiomycetes in different ecosystems. At present, these isolates are being used in our various lab experiments and can be useful in different industrial and bioremediation applications.

Keywords

Basidiomycetes; guaiacol; geographic regions; saprophytic

Introduction

In forest ecosystems, various combinations of vegetation cover consistently produce huge amounts of organic matter playing a dominant role in soil structure and fertility. A very large number of microorganisms are involved in litter decomposition under different environmental conditions. Saprophytic Basidiomycetes are important and dominant recyclers of plant wastes in soil. This fungal group is the main producer of lignin-degrading enzymes such as manganese peroxidase and laccase [1]. Fungal

laccases are of great interest due to their higher redox potential for lignin and polyphenol degradation, potential use in many industrial applications in paper, textile, food, and pharmaceutical sectors, and in the degradation of aromatic pollutants causing environmental problems [2]. Exploring novel laccases with different substrate specificities and enhanced stabilities is desirable for industrial applications, besides developing an effective and economic production medium with high yields to enhance their utility [3]. Therefore, there is a need to find new laccase producers from different geographic and environmental conditions.

The state of Michoacán is among the five states with the greatest biodiversity in Mexico [4], partly due to its geographical location in the transition zone between the Nearctic and Neotropical regions, which generates a variety of ecosystem types. To date, studies have been performed to increase the awareness about the diversity of fungi in the state of Michoacán, with a major focus on the Basidiomycota group. Significant classical taxonomic work within this group has been carried out in various ecosystems of this region in the past. Discovery of novel laccase-producing fungi is important to improve sources of more active, thermostable, or acid tolerant enzymes for industrial applications.

Previous studies exhibited that various ecosystems have the potential to warrant an exploration of laccase-producing fungi due to the fungal diversity and geographic position of this region. Therefore, this study was designed to complement classical taxonomic work with biotechnology by building a gene bank and an ex situ collection of long-term vegetative (mycelia) or asexual (spores) propagation structures. In this study, isolation, screening, and molecular identification of potential laccase-producing native saprophytic Basidiomycetes species from different natural forest areas were performed.

Material and methods

Site information and samples collection

For the collection of soil samples, different areas were selected in the state of Michoacán, Mexico. The sites were chosen based on their ecological characteristics and geographical location. The information on the sampling sites appears in Tab. 1.

Soil samples were collected from different habitats and three to four replicate plots of each habitat were sampled. Soil cores of 2.5-cm diameter were taken from 25-cm depth after the litter layer was removed. Sample for each location was pooled from four or more cores per site. Vegetative mycelial aggregates in soil were also collected separately for direct isolation from known/unknown Basidiomycetes species. The samples were marked with information such as collection numbers along with names, sampling location, and date of collection. Mycelial samples were wrapped in aluminum foil, brought to the laboratory, and stored in a refrigerator at 4°C for further study.

Tab. 1 Sample collection sites in the state of Michoacán, Mexico.

Region and location	Type of vegetation	Coordinates	Altitude (m)
Ejido La Ampliación; Atécuaro, Morelia	Forest of pine and pine-oak	N 19°33′59.4″, W 101°07′48.2″	2,350
Parque Nacional Insurgente José María Morelos y Pavón (Km 23)	Pine-oak forest and oak forest	N 19°39′45.7″, W 101°00′19.7″	2,150
Presa La Gachupina, Municipio de Jerécuaro (Ciudad Hidalgo)	Forest of pine and cedar	N 19°49′23.63″, W 100°39′10.35″	2,950
Parque Nacional "Barranca del Cupatitzio" (PNBC), Uruapan	Pine-oak forest	N 19°26′16.63″, W 102°06′57.22″	2,090
Ichaqueo, Morelia	Forest of pine, pine-oak and mesophyll mountain	N 19°34′11.04″, W 101° 8′45.0″	2,200
Tarímbaro	Mix vegetation	N 19°47′0″, W 101°8′0″	1,888

Isolation of fungal isolates with the help of guaiacol as an indicator

Isolation of basidiomycetes from soil samples. Selective soil particle-washing technique [5] was employed for the isolation of Basidiomycetes from soil samples. Approximately 5–6 g of each fresh soil sample was added into 500 mL of sterile 0.1% (wt/vol) sodium pyrophosphate in a 1-L beaker. Soil solution was gently stirred for 1 h with glass rod at room temperature (25 ±2°C) to disperse soil clumps and colloids. The soil suspension was passed through a stack of 20-cm soil sieves of 250 mm (No. 60) and 53 mm (No. 270) mesh. Particles on the 53-mm mesh were washed thoroughly, organic particles were collected and added into sterile distilled water. A 0.4 mL suspension of organic particles was spread onto petri dishes with 2% Potato Dextrose Agar [PDA; 200 g/L potato, 20 g/L glucose (Sigma), 20 g/L agar (Sigma)] medium [6,7] containing guaiacol (Sigma), benomyl (Sigma), and antibiotic (tetracycline) at concentrations of 200 μl/L, 0.32 g/L, and 500 mg/L, respectively. The dishes were incubated at 25 ±2°C. After 8–10 days, the petri dishes were scanned for colonies that caused reddening of the guaiacol by the action of laccases (Fig. 1). These colonies were also examined microscopically for the presence of conidia or clamp connections. Colonies showing laccase activity (reddening in medium) and characteristics of basidiomycetes (clamp connections) were transferred onto 2% PDA to obtain pure cultures.

Isolation of Basidiomycetes cultures from mycelial aggregates. Clumps of vegetative mycelial aggregates (0.5–1 cm) from soil were washed thoroughly with running tap water to remove soil particles from mycelial samples, then washed three times with sterile distilled water, placed on sterile filter paper, inoculated aseptically onto 2% PDA medium [supplemented with tetracycline (0.5 mg/mL), guaiacol (0.2 uL/mL), and benomyl (0.32 mg/mL)], and incubated at 25 ±2°C for 8–10 days. Each fungal colony was examined for reddening or browning in medium due to laccase activity as mentioned above. Pure isolates were transferred onto 2% PDA medium for further study. The fungal species were also submitted to a long-term preservation for future research: the mycelial pieces were stored in cryotubes with 30% sterilized glycerol at −80°C and also preserved in sterile water at the Institute.

Molecular identification of Basidiomycete isolates – isolation of genomic DNA. To obtain a high concentration of genomic DNA from fungal isolates, pure mycelium (500 to 1,000 mg) of each strain was harvested into a separate 1.5 mL microtube containing 0.20 g of E-matrix lysis (a mixture of ceramic and silica spheres of diameter 1.2 at 1.6 mm and from 0.074 to 0.150 mm, respectively; MP Biomedicals, USA) and 500 μL of lysis buffer containing 100 mM Tris HCl (pH 8.0) was added; 100 mM EDTA (pH 8.0), 20 mM NaCl, and 2% SDS were also added to the tube. The samples were subjected to stirring at 6.0 m/s for 35 s on the Fast Prep-24 homogenizer (MP Biomedicals, USA), followed by incubation in a thermomixer (Eppendorf, USA) at 60°C for 30 min with shaking for 5 s at 10-min intervals. The sample mixture was centrifuged at 10,000 rpm for 5 min, supernatant was transferred to a sterile tube and an equal volume of phenol:chloroform (1:1) mixture was added. It was then vortexed for 3 min and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a new sterile tube, two volumes of cold absolute isopropanol were added, and incubated at −20°C for 1 h. The DNA pellet was recovered by centrifugation (10,000 rpm for 10 min), washed with cold ethanol at 70% (v/v), dried at 37°C for the necessary time, dissolved in 25 μL of water, and stored at −20°C until use. The RNA was removed by enzymatic digestion with RNAse (10 mg/mL). Two microliters of RNAse stock was added to the aqueous phase with DNA and incubated for 30 min at 37°C. Once the incubation time was over, the DNA was precipitated by adding the same volume of cold isopropanol, shaken gently, and incubated at −20°C for 1 h or overnight. The sample was centrifuged at 10,000 rpm for 5 min, the supernatant was decanted, and then the pellet was washed with 250 μL of 70% ethanol and dried at room temperature for 15 min. Finally, the pellet was resuspended in 25 μL of sterile deionized distilled water. The DNA obtained was stored at −20°C for later use.

In order to assess the quality of the DNA obtained, a 1% agarose gel electrophoresis stained with ethidium bromide (final concentration of 1 μg/mL) was performed in TAE buffer (working solution: 40 mM Tris, 1 mM EDTA, glacial acetic acid 1.2 μL/mL,

pH 8.0) at 100 V. DNA quantification was performed by Nanodrop-2000c (Thermo-Scientific, USA) spectrophotometer. A working DNA concentration of 25 ng/μL was used for PCR reactions.

Molecular identification of Basidiomycete isolates – amplification of internal transcribed spacer (ITS) region and sequencing. The ITS1-5.8S-ITS2 genomic region of each isolate was amplified from genomic DNA by using the forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and the reverse primer ITS4 (5′-TCCTC-CGCTTATTGATATGC-3′) [8]. Each PCR reaction was carried out in 25 μL solution containing 2.0 mM MgCl$_2$, 0.2 mM of each primer, 0.2 mM of each dNTP, 0.1 mg of bovine serum albumin (BSA), and 0.01 U/μL of Taq DNA polymerase (Invitrogen, Life Technologies, USA). An aliquot of 25 ng of DNA was used in each PCR reaction. The amplification protocol consisted of an initial denaturation step of 5 min at 95°C, followed by 35 cycles of amplification as follows: 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. A final extension of 10 min was performed at 72°C. After amplification, an aliquot of 5 μL was analyzed by electrophoresis on 1.5% TAE agarose gel, visualized under UV light, and PCR products were compared with the molecular size standard 1 kb plus DNA ladder (Invitrogen, USA). Purification and sequencing of amplified DNA fragments were performed at Elim Biopharmaceuticals, Inc. (USA).

Molecular identification of Basidiomycete isolates – sequence analysis of the ITS region. The quality of the obtained ITS sequences was analyzed using the Chromas Lite 2.0 software (https://technelysium.com.au/wp/chromas). High quality ITS sequences longer than 400 bp were subjected to BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to find the sequences showing maximum identity with those deposited in GenBank (Tab. 3). ITS sequences regions from GenBank showing the highest identity with the sequences obtained from the isolates in this study were retrieved and subjected to multiple alignment analysis using the Clustal W program of BioEdit. The phylogenetic relationships between laccase-producing fungi sequences were determined by MEGA5 [9] software from the multiple alignment files. The best evolutionary model for each group of sequences was determined and the phylogenetic trees were constructed using the neighbor-joining (NJ) criterion. A bootstrap analysis was performed using 1,000 replicas. Information about the fungal taxonomic hierarchical levels was obtained from the databases MycoBank (http://www.mycobank.org) and Index Fungorum (http://www.indexfungorum.org).

Results

This study has been carried out to identify potential laccase-producing saprophytic Basidiomycetes from soil samples to use in industrial processes. The samples were collected from different ecological and geographical areas of Michoacán State, Mexico.

Isolation and screening of laccase-producing fungal isolates

In total, 15 Basidiomycete colonies were successfully isolated from soil and vegetative mycelial aggregates. During isolation and screening experiments, fungal colonies formed reddish-brown halo/coloration under or around the colony on 0.02% guaiacol supplemented 2% PDA medium, and were identified as laccase-positive and purified for further molecular identification (Fig. 1). Fungal colonies without laccase activities were also purified for conservation purposes. Among the 171 saprophytic fungal isolates, only 15 were identified as Basidiomycetes with strong laccase activity and exhibited very intense reddish-brown halo/coloration in medium plates. High number of laccase-producing Basidiomycete isolates were isolated from soil samples, collected from Parque Nacional José Ma. Morelos, Km 23, Charo, Michoacán, Mexico (Tab. 2).

Laccase producing isolates were identified as Basidiomycetes on the basis of clamp connections or conidia production. However, a variation had been found in the laccase activity of different isolates on medium supplemented guaiacol (Fig. 2, Fig. 3).
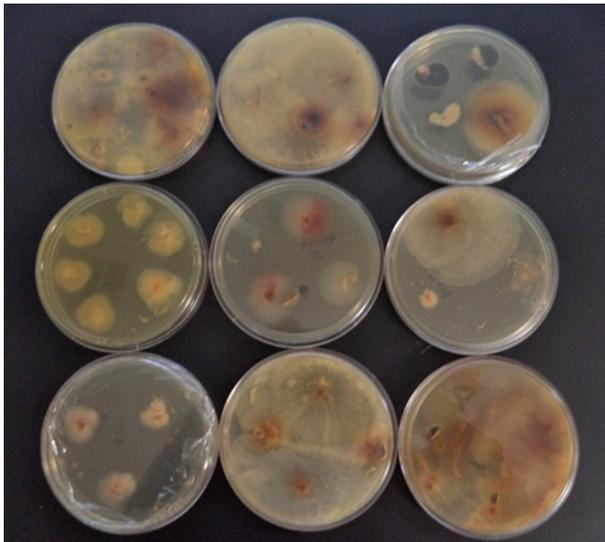
Fig. 1   Screening of fungal isolates from soil and vegetative mycelia samples for laccase activities. Reddish brown coloration on the reverse sides of fungal colonies indicate oxidation of guaiacol in medium due to laccase production.
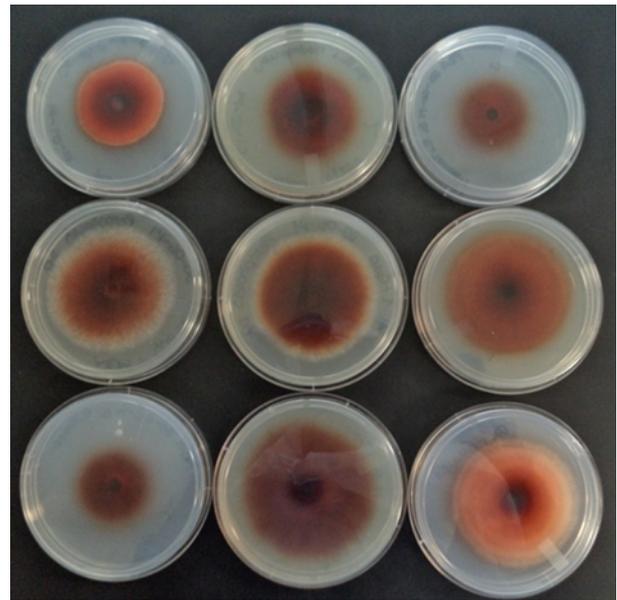
Fig. 2   Purified basidiomycete isolates with strong laccase activity (reverse view of fungal isolates).

Tab. 2   List of laccase positive saprophytic Basidiomycete isolates from different ecological areas in Michoacán State, Mexico.

Sr. No.	Isolate No.	Location	Type of sample	Guaiacol activity*
1	CMU02-13	Parque Nacional, José Ma. Morelos, Km 23, Charo	Mycelial aggregation	+++++
2	CMU23-13	Parque Nacional, José Ma. Morelos, Km 23, Charo	Mycelial aggregation	+++++
3	CMU25-13	Parque Nacional, José Ma. Morelos, Km 23, Charo	Mycelial aggregation	+++++
4	CMU35-13	Parque Nacional, José Ma. Morelos, Km 23, Charo	Mycelial aggregation	++++
5	CMU37-13	Parque Nacional, José Ma. Morelos, Km 23, Charo	Mycelial aggregation	+++++
6	CMU43-13	Parque Nacional, José Ma. Morelos, Km 23, Charo	Mycelial aggregation	+++++
7	CMU45-13	Parque Nacional, José Ma. Morelos, Km 23, Charo	Soil	+++++
8	CMU47-13	Parque Nacional, José Ma. Morelos, Km 23, Charo	Mycelial aggregation	+++++
9	CMU55-13	Ejido La Ampliación; Atécuaro, (Morelia)	Mycelial aggregation	+++++
10	CMU67-13	Ejido La Ampliación; Atécuaro, (Morelia)	Soil	++++
11	CMU84-13	Ejido La Ampliación; Atécuaro, (Morelia)	Soil	+++++
12	CMU85-13	La Posta Veterinaria y Zootecnia (Tarímbaro)	Soil	+++++
13	CMU86-13	La Posta Veterinaria y Zootecnia (Tarímbaro)	Soil	++++
14	CMU87-13	La Posta Veterinaria y Zootecnia (Tarímbaro)	Mycelial aggregation	+++++
15	CMU88-13	La Posta Veterinaria y Zootecnia (Tarímbaro)	Leaf debris in soil	++++

* Key: +++++ – excellent; ++++ – very good; +++ – good; ++ – average; + – poor; - – none.

Molecular identification of laccase-positive isolates

In total, fifteen Basidiomycetes fungi isolates were recovered, which were closely related to the genera *Bjerkandera*, *Coriolopsis*, *Ganoderma*, *Hexagonia*, *Irpex*, *Limonomyces*, *Psathyrella*, *Peniophora*, *Phlebia*, *Phlebiopsis*, *Trametes*, and *Trichaptum* and family Corticiaceae (Tab. 3). However, no specific phylotype could be isolated as a common representative of all sampling sites. The phylogenetic analysis of each isolate was carried out to show the relationship between individual sequences and the closest relatives retrieved from GenBank database. Results showed that purified isolates belonged to eight families: Corticiaceae, Ganodermataceae, Hymenochaetaceae, Meruliaceae,
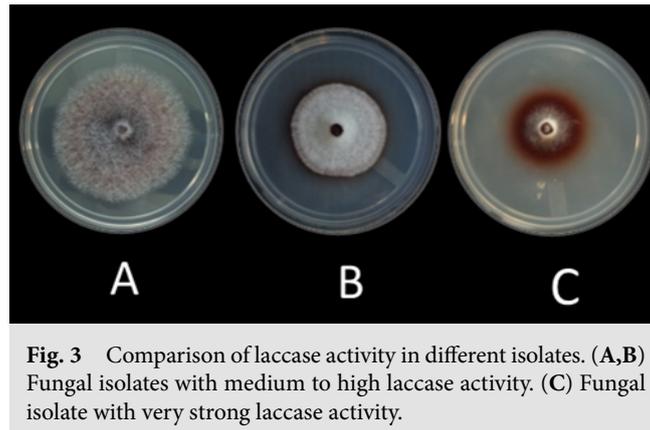
Fig. 3 Comparison of laccase activity in different isolates. (**A,B**) Fungal isolates with medium to high laccase activity. (**C**) Fungal isolate with very strong laccase activity.

Tab. 3 Isolate code, number of isolates, maximum identities in GenBank sequences, number of bp analyzed, identification, and GenBank accession number of saprophytic basidiomycetes fungi in different ecosystems in Michoacán, Mexico.

Isolation code	Isolation source	Collection site	Maximum identity (%)	No. of base pairs analyzed	Proposed identification	Gene bank accession No.
CMU02-13	Mycelial aggregation	Atécuaro (pine-oak forest)	99	589	*Ganoderma curtisii*	MH347284
CMU23-13	Mycelial aggregation	Atécuaro (pine-oak forest)	99	575	*Bjerkandera adusta*	MH347285
CMU25-13	Mycelial aggregation	Atécuaro (pine-oak forest)	87	624	Species belong to Corticiaceae	MH347286
CMU35-13	Mycelial aggregation	Km 23, Charo (pine-oak forest)	96	589	*Hexagonia* sp.	MH347287
CMU37-13	Mycelial aggregation	Km 23, Charo (pine-oak forest)	100	392	*Trametes versicolor*	MH347288
CMU43-13	Mycelial aggregation	Km 23, Charo (pine-oak forest)	99	612	*Trichaptum biforme*	MH347289
CMU45-13	Soil	Km 23, Charo (pine-oak forest)	97	419	*Hexagonia* sp.	MH347290
CMU47-13	Mycelial aggregation	Km 23, Charo (pine-oak forest)	98	602	*Phlebiopsis* sp.	MH347291
CMU55-13	Mycelial aggregation	Atécuaro (pine-oak forest)	99	604	*Phlebia acerina*	MH347293
CMU67-13	Soil	Atécuaro (pine-oak forest)	99	475	*Trametes versicolor*	MH347296
CMU84-13	Soil	Atécuaro (pine-oak forest)	99	563	*Irpex lacteus*	MH347298
CMU85-13	Soil	Tarímbaro (mesophyll mountain forest)	97	647	*Limonomyces* sp.	MH347299
CMU86-13	Soil	Tarímbaro (mesophyll mountain forest)	99	639	*Psathyrella candolleana*	MH347300
CMU87-13	Mycelial aggregation	Tarímbaro (mesophyll mountain forest)	97	591	*Coriolopsis rigida*	MH347301
CMU88-13	Leaf debris in soil	Tarímbaro (mesophyll mountain forest)	<96	583	*Peniophora* sp.	MH347302

Peniophoraceae, Phanerochaetaceae, Polyporaceae, and Psathyrellaceae from five different orders (Agaricales, Corticiales, Hymenochaetales, Polyporales, and Russulales).

The ITS sequence of CMU02-13 isolate displayed a sequence identity of 99% with several sequences deposited in GenBank; 99% with *Ganoderma curtisii* (MG966318, MH165297) and *G. meredithae* (KY70888) from the USA. ITS sequence alignment of CMU02-13 with BLAST sequences exhibited two nucleotide differences compared to the sequence of *G. meredithae* (KY70888), but no difference with *G. curtisii* (MG966318, MH165297. Phylogenetic relationships between *Ganoderma* species showed that isolate CMU02-13 formed a monophyletic group with *G. curtisii* (Fig. 4).
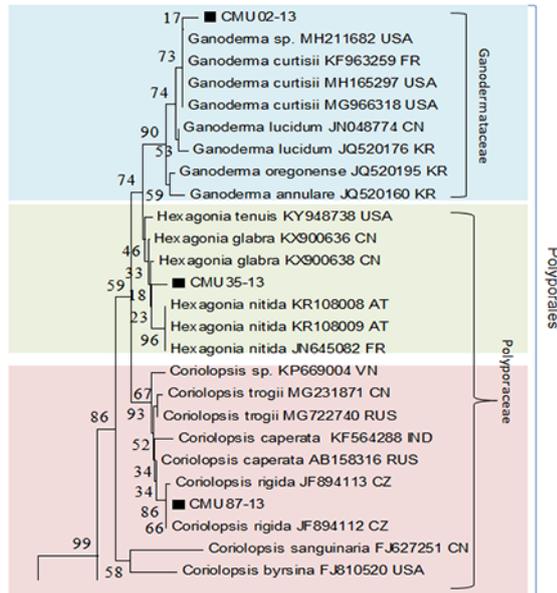
The CMU23-13 isolate sequences showed 99% identities with published ITS sequences [KC176310, KC176335 (Canada); FJ608590 (Czech Republic)] of *Bjerkandera adusta* and *Thanatephorus cucumeris* isolates [FR670341 (France); EF155506 (Germany); AF455463 (Austria)] from GenBank. Sequence analysis showed that CMU23-13 presented five nucleotide differences to isolates of *B. adusta* (KC176335, FJ608590). Various NCBI sequences of *B. adusta* isolates were added to analyze relationships of rDNA sequences of CMU23-13 with other polypore representatives. Phylogenetic tree analysis showed that *B. adusta* isolates formed one clade with a strong bootstrap support. The clade of *B. adusta* was further divided into two subclades depending on the origin of isolates. Fungal isolate CMU23-13 formed a monophyletic group with two different isolates (KC176335, KC176310) of *B. adusta* from Canada; however, CMU23-13 has a distinct position in this group (Fig. 4).
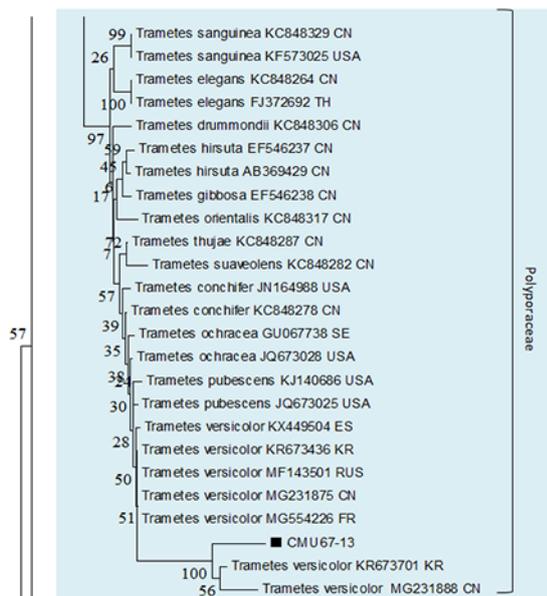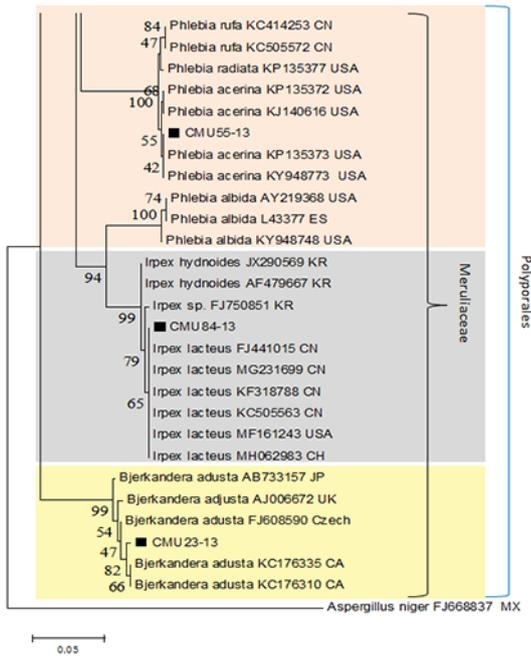
The fungal isolate CMU25-13 sequences showed 87% identity with Corticiaceae sp.4 [JN225959 (New Zealand)] with eighty nucleotide differences and 82% similarity with Corticiaceae sp.2 [JN225960 (New Zealand)]. Phylogenetic analysis with closely related sequences from NCBI and other members of order Corticiales showed that CMU25-13 is highly similar to Corticiaceae sp.4 sequences (Fig. 5).
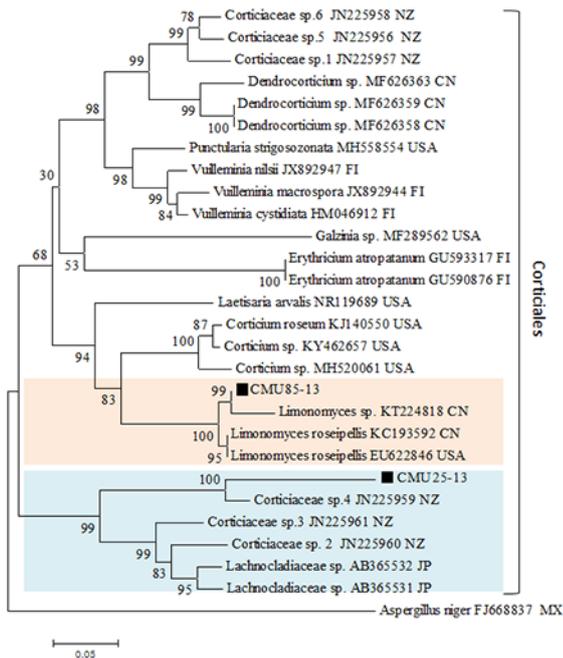
Sequence analysis of CMU35-13 displayed a 25-nucleotide difference to the NCBI sequences of *Hexagonia glabra* [KX900637 (China)] and [KP013019 (Australia)] with a similarity of 96%. In addition, CMU35-13 also showed a 95% sequence homology with *Daedaleopsis* spp. [KF541329, KF541330 (Australia)]. However, in the phylogenetic analysis, CMU35-13 was clustered along with *H. glabra* isolates, but placed in a separate position (Fig. 4). According to BLAST analysis, CMU45-13 displayed an identity of 97% and >20 nucleotide difference with two isolates of *H. glabra* [KX900637 (China); KP013019 (Australia)]. However, alignment results showed 100% similarity between CMU45-13 and CMU35-13.
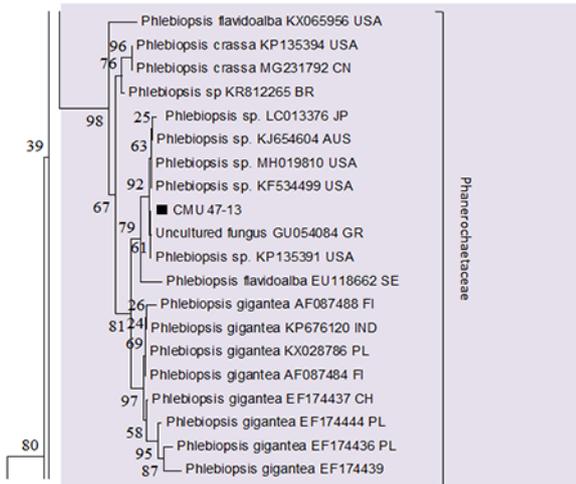
In the case of CMU67-13, nucleotide similarities of 99% [X449504(Spain); MF143501 (Russia); MG554226 (France)] and 95% [MG231888 (China); KR673701 (Korea)] were observed with different isolates of *T. versicolor* in GenBank. In phylogenetic analysis, an internal arrangement of two different subclades was observed within the *T. versicolor* clade. Isolate CMU67-13 was clustered along with isolates MG231888 and KR673701, but in a distinct position (Fig. 4). Similarly, the ITS sequence of CMU37-13 displayed 100% identity with many *T. versicolor* sequences [i.e., MH211987 (USA); KX218391 (Germany); MG554226 (France)] in NCBI without any difference in nucleotides. This isolate has not been included in the phylogenetic tree.

Figure (phylogenetic trees):

Polyporales — Ganodermataceae:
17 — CMU 02-13
Ganoderma sp. MH211682 USA
73 Ganoderma curtisii KF963259 FR
74 Ganoderma curtisii MH165297 USA
Ganoderma curtisii MG966318 USA
90 Ganoderma lucidum JN048774 CN
53 Ganoderma lucidum JQ520176 KR
74 Ganoderma oregonense JQ520195 KR
59 Ganoderma annulare JQ520160 KR

Polyporaceae:
Hexagonia tenuis KY948738 USA
Hexagonia glabra KX900636 CN
46 Hexagonia glabra KX900638 CN
33 CMU 35-13
59 18 Hexagonia nitida KR108008 AT
23 Hexagonia nitida KR108009 AT
96 Hexagonia nitida JN645082 FR

Coriolopsis sp. KP669004 VN
67 Coriolopsis trogii MG231871 CN
93 Coriolopsis trogii MG722740 RUS
86 52 Coriolopsis caperata KF564288 IND
Coriolopsis caperata AB158316 RUS
34 Coriolopsis rigida JF894113 CZ
34 CMU 87-13
86 Coriolopsis rigida JF894112 CZ
66 Coriolopsis sanguinaria FJ627251 CN
99 58 Coriolopsis byrsina FJ810520 USA

Polyporaceae:
99 Trametes sanguinea KC848329 CN
26 Trametes sanguinea KF573025 USA
Trametes elegans KC848264 CN
100 Trametes elegans FJ372692 TH
Trametes drummondii KC848306 CN
97 59 Trametes hirsuta EF546237 CN
45 Trametes hirsuta AB369429 CN
17 Trametes gibbosa EF546238 CN
Trametes orientalis KC848317 CN
72 Trametes thujae KC848287 CN
7 Trametes suaveolens KC848282 CN
57 Trametes conchifer JN164988 USA
Trametes conchifer KC848278 CN
39 Trametes ochracea GU067738 SE
35 Trametes ochracea JQ673028 USA
38 Trametes pubescens KJ140686 USA
30 Trametes pubescens JQ673025 USA
Trametes versicolor KX449504 ES
28 Trametes versicolor KR673436 KR
50 Trametes versicolor MF143501 RUS
Trametes versicolor MG231875 CN
51 Trametes versicolor MG554226 FR
CMU 67-13
100 Trametes versicolor KR673701 KR
56 Trametes versicolor MG231888 CN
57

Phanerochaetaceae:
Phlebiopsis flavidoalba KX065956 USA
96 Phlebiopsis crassa KP135394 USA
76 Phlebiopsis crassa MG231792 CN
Phlebiopsis sp KR812265 BR
98 25 Phlebiopsis sp. LC013376 JP
63 Phlebiopsis sp. KJ654604 AUS
Phlebiopsis sp. MH019810 USA
92 Phlebiopsis sp. KF534499 USA
67 CMU 47-13
79 Uncultured fungus GU054084 GR
61 Phlebiopsis sp. KP135391 USA
Phlebiopsis flavidoalba EU118662 SE
26 Phlebiopsis gigantea AF087488 FI
81 24 Phlebiopsis gigantea KP676120 IND
69 Phlebiopsis gigantea KX028786 PL
Phlebiopsis gigantea AF087484 FI
97 Phlebiopsis gigantea EF174437 CH
58 Phlebiopsis gigantea EF174444 PL
95 Phlebiopsis gigantea EF174436 PL
87 Phlebiopsis gigantea EF174439
39 80

Fig. 4 Phylogenetic relationship of ■CMU25-13 and ■CMU85-13 with species in Corticiales inferred from ITS sequences using NJ analysis.

Fig. 5 Phylogenetic relationship of ■CMU25-13 and ■CMU85-13 with species in Corticiales inferred from ITS sequences using NJ analysis.

The BLAST analysis of CMU43-13 exhibited 99% sequence identity to published NCBI sequences of *Trichaptum biforme* [JN601154, MF773616 (USA)]. A difference of five nucleotides was identified between CMU43-13 and JN601154 sequences. Isolate CMU43-13 sequences showed a strong phylogenetic relationship with those of *T. biforme* isolates (Fig. 6).

ITS sequence of CMU47-13 showed 99% [GU054084 (Germany)] and 98% [KF534499, KP135391 (USA)] identities with GenBank ITS sequences of uncultured fungal isolates and *Phlebiopsis* sp. The CMU47-13 sequences exhibited three nucleotide differences from GU054084. Phylogenetic tree analysis showed that *Phlebiopsis* spp. and CMU47-13 isolate form a monophyletic group (Fig. 4). The CMU55-13 sequence exhibited 99% identity to *Phlebia acerina* [KP135373, KY948773 (USA)] and *Echinodontium taxodii* [AF455458 (Austria); AB369433 (China)] sequences deposited in GenBank. However, phylogenetic analysis showed that isolate CMU55-13 belonged to a separate subclade with other three isolates of *P. acerina* (Fig. 4).

The phylotype CMU84-13 displayed 99% identity with ITS sequences of *Irpex lacteus* [FJ441015, MG231699, and KF318788 (China)] in GenBank. In the Clustal W analysis with other *Irpex* species, the phylotype CMU84-13 presented a difference of five nucleotides from three strains of *I. lacteus* (FJ441015, MG231699, and KF318788). Fungal isolate CMU84-13 formed a distinct position within the monophyletic group of *I. lacteus* (Fig. 4).

ITS of CMU85-13 displayed 97% identity with published ITS sequences of *Limonomyces roseipellis* [KC193592 (China); EU622846 (USA)] and genus *Laetisaria* [*Laetisaria arvalis* – AM262443 (Spain)]. However, in the phylogenetic analysis, the phylotype CMU85-13 presented a difference of 18 nucleotides from two strains of *L. roseipellis* (KC193592, EU622846). The CMU85-13 sequences showed more than a hundred nucleotide difference from *Laetisaria* species. Phylogenetic tree analysis showed that the isolates of *L. roseipellis* (KC193592 and EU622846) separated into one distinct group at 100% identity with CMU85-13 subclade (Fig. 5). However, species of *Laetisaria* formed a distinct position in the phylogenetic tree with respect to the outgroup.

The phylotype CMU86-13 exhibited an identity of 99% with various sequences of *Psathyrella candolleana* isolates [AF345810 (Korea); DQ389720 (Sweden); EU520251 (China)] with a nucleotide difference of five. The phylogenetic tree was generated from rDNA sequences of various isolates of *P. candolleana* and some other members of Agaricales. The results showed that CMU86-13 is well positioned in a monophyletic clade with other *P. candolleana* isolates (Fig. 7).

BLAST search exhibited sequence similarity of up to 99% between CMU87-13 and various isolates of *Coriolopsis* species. Sequence analysis of CMU87-13 displayed 1–15 nucleotide difference (98–99% nucleotide similarity) to isolates of *C. rigida* [JF894112, JF894113 (Czech Republic)]. In BLAST analysis, *C. caperata* [AB158316 (Russian Federation)] also showed a similarity of 97% with CMU87-13. However, the phylogenetic tree demonstrated a monophyletic clade of *C. rigida* (JF894112, JF894113) and CMU87-13 (Fig. 4).

The sequence of CMU88-13 displayed 99% identity with published ITS sequences of uncultured fungal isolates [JF289101, JF289100 (Germany)] and unpublished sequence of genus *Peniophora* sp. [KC771452 (Ecuador)]. However, various *Peniophora* species [HQ604854 (Canada): JX434676 (China): JX046435 (Russia)] also showed <96% identity with CMU88-13 sequences. Phylogenetic analysis showed that CMU88-13 has
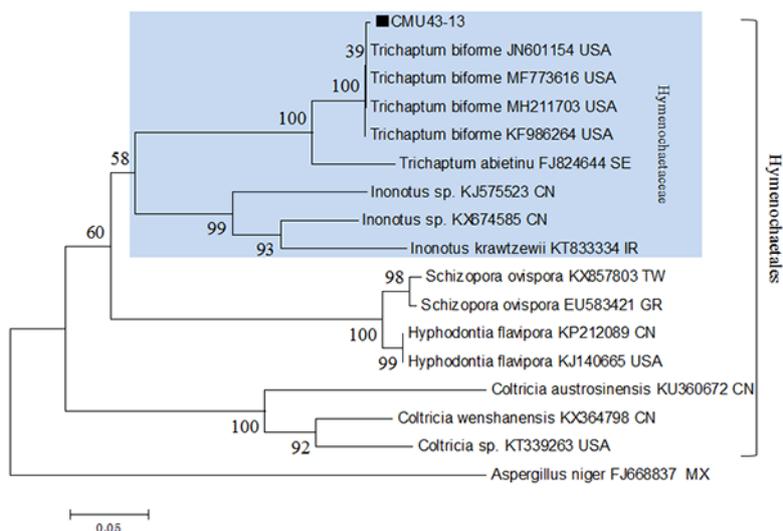
Fig. 6 NJ analysis of ITS sequence data of ■CMU43-13 in Hymenochaetaceae.
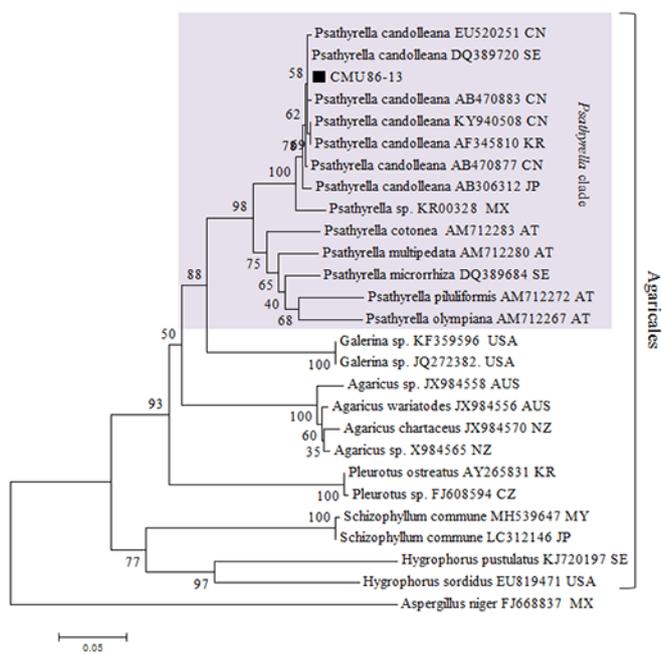
Fig. 7 Phylogenetic tree based on NJ analysis is showing the relationship of ■CMU86-13 with other *Psathyrella* species.

strong affinity to be positioned separately in a distinct clade along with uncultured fungal (JF289101) and *Peniophora* sp. (KC771452) isolates (Fig. 8).

Discussion

Saprophytic fungi are potential sources of biodegradation processes in natural ecosystems due to their versatile enzyme activities. Basidiomycetes are the best laccase-producer and are also potential lignin degraders, mainly in low temperature environments. In the present investigation, various laccase-producing species of Basidiomycetes have been isolated from different ecosystems situated in distinct geographic areas of Michoacán, Mexico. These characteristics support fungal diversity and efficiency for laccase activities in various environmental conditions. In this study, we obtained only 15 isolates of 13 genera from 19 soil samples collected from different ecosystems. Each genus was represented by a single or two isolates. No attempt was made to identify isolates that did not give positive reactions for laccase even if they were capable to grow on guaiacol-benomyl agar. However, it has been reported that many basidiomycetes can grow on guaiacol-benomyl agar without coloration in medium [10]. This indicates the inadequacy of sampling/isolation and the potential existence of diversity in laccase-producing Basidiomycetes in these soils. More samples or modified isolation techniques would be undoubtedly helpful in obtaining more isolates and hence a wider diversity.

Reddish brown halo/coloration below colonies were due to the oxidative polymerization of guaiacol in the presence of extracellular laccases produced by the fungal isolate. Various color indicators are used for the visual recognition of laccase enzyme as a direct method for microbial screening of laccase activity [11]. As a colorimetric indicator, guaiacol showed very strong ability to facilitate the growth and the isolation of laccase-producing fungi. The color reaction with guaiacol is easily detectable, and helpful for active screening of laccase-positive isolates [12]. Another study supports that plate-method with indicator compounds is an efficient and simple method for screening bio-prospective fungi with lignolytic enzymes for industrial applications [13]. The
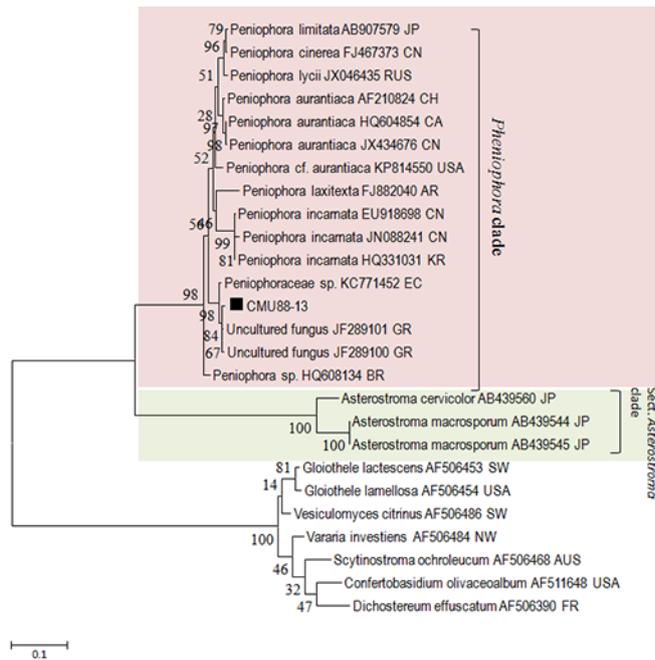
Fig. 8 Phylogenetic tree obtained from NJ analysis of ITS sequence data of taxa in Peniophoraceae (belonging to Russulales). Symbol ■ indicates the isolate in this study, whereas other sequences represent reference data obtained from GenBank.

intensity and diameter of the halo indicate the quality of the laccase enzyme secreted by fungus (Fig. 3). Similar studies were carried out to isolate new fungal isolates from soil for laccase activity [14–16].

Basidiomycete isolates were identified by using the sequencing of the ITS region between the rRNA genes 18S and 26S with a sequence length between 392 and 647 bp. *Ganoderma curtisii* (CMU02-13), *B. adusta* (CMU23-13), *H. tenuis* (CMU35-13), *T. versicolor* (CMU37-13 and CMU67-13), *T. biforme* (CMU43-13), *Phlebia acerina* (CMU55-13), *I. lacteus* (CMU84-13), *L. roseipellis* (CMU85-13), *P. candolleana* (CMU86-13), and *C. rigida* (CMU87-13) had sequences closely related to the sequences that are deposited in the GenBank database. Due to the limited information in GenBank, the isolates Corticiaceae sp., (CMU25-13), *Hexagonia* sp., (CMU45-13) *Phlebiopsis* sp., (CMU47-13), and *Peniophora* sp. (CMU88-13) were identified only on the genus level.

The species isolated in the present study are important from an industrial point of view. Several of them are reported here for the first time as laccase-producing species of Corticiaceae, *P. candolleana*, and *Peniophora* sp., from Mexico.

The present study showed that different ecological ecosystems in the state of Michoacán, have a great potential that warrants exploration of new fungal species producing laccase and other industrial important biochemicals. Fungal isolates with laccase activity can be used further in biotechnological and bioremediation applications. The molecular identification of 15 laccase-positive saprophytic fungal isolates is an important step for further investigation in industrial applications. Furthermore, optimization of the growth conditions of fungal isolates will be important to enhance laccase production for industrial processes in Mexico. While current studies on laccase-producing Basidiomycetes offer very limited information, further investigation can be performed to improve our knowledge on laccase-producing species in different ecosystems in Mexico.

Acknowledgments

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