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ORIGINAL PAPER

White rot Basidiomycetes isolated from Chiloé National Park in Los Lagos region, Chile

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Abstract Wood decomposition is an important component in forest ecosystems but information about the diversity of fungi causing decay is lacking. This is especially true for the temperate rain forests in Chile. These investigations show results of a biodiversity study of white-rot fungi in wood obtained from Chiloé National Park in Los Lagos region, Chile. Culturing from white-rotted wood followed by sequencing of the complete internal transcribed spacer region of the ribosomal DNA (rDNA) or partial large subunit region of the rDNA, identified 12 different species in the Basidiomycota. All of these fungi were characterized as white rot fungi and were identified with a BLAST match of 97 % or greater to sequences in the GenBank database. Fungi obtained were species of *Phlebia, Mycoacia*, *Hyphodontia*, *Bjerkandera*, *Phanerochaete, Stereum*, *Trametes*, and *Ceriporiopsis.* This report identifies for the first time in Chile the species *Ceriporiopsis subvermispora*, *Hyphodontia radula,*

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Phlebia radiata, Phanerochaete affinis, Peniophora cinerea, Stereum gausapatum, Phlebia setulosa and Phanerochaete sordida. Scanning electron microscopy was used to characterize the type of decay caused by the fungi that were isolated and a combination of selective lignin degraders and simultaneous white rot fungi were found. Fungi that cause a selective degradation of lignin are of interest for bioprocessing technologies that require modification or degradation of lignin without cellulose removal.

Keywords Wood · DNA · Chiloé · Biodiversity · White rot fungi · Biodegradation

Introduction

Wood can be attacked by a variety of microorganisms, but fungi in the Basidiomycota are considered the main decomposers in terrestrial ecosystems (Akhtar et al. 1997; Highley and Dashek 1998; Urairuj et al. 2003). These organisms are distributed widely in forests throughout the world, and species as well as strains vary considerably in terms of their cellulolytic and ligninolytic capabilities (Blanchette 2003; Tortella et al. 2008). Among the different types of fungi that attack wood, white-rot fungi are most common in deciduous forests (Eriksson et al. 1990) and their degradative processes have been of great interest for their ability to degrade all cell wall components including lignin (Akhtar et al. 1997; Eriksson 1981; Kirk and Cullen 1998; Highley and Dashek 1998; Martínez et al. 2005; Dashtban et al. 2010; Halis et al. 2012; Leisola et al. 2012). The ability of some white rot fungi to selectively attack lignin and other complex compounds make them useful for biotechnological uses, such as bioremediation, biobleaching of pulp, biopulping and pretreating biomass for bioenergy production (Blanchette 1991; Highley and Dashek 1998; Tortella et al. 2008).

Butin and Peredo (1986) and Furci (2008) have indicated that Chile is a relatively rich country in terms of fungal flora. However, only about 3,300 fungi species are known from Chile, and additional studies are needed to get a more comprehensive view of the fungi present (Gamundi and Horak 1993; Lazo 1995; Lazo 2001; Minter and Peredo 2006; Furci 2007).

According to Gamundi (2003), the most important collection of fungi, based on bibliographic data published, was conducted by Mujica et al. (1980) in his work called 'Flora Fungosa Chilena'. Since that time, some additional studies have been completed on the identification of species and enzymatic capacity of fungi in Chile (Lazo 1996; Valenzuela et al.1996; Lanfranco et al. 2003; Valenzuela and Barrera 2001). Several studies have also been carried out in the Southern region of Chile to identify fungi associated with an unusual degradation pattern called "palo blanco" which means "pure white decayed wood" with an exceedingly high degree of delignification and "palo podrido" which means "rotten wood" and is decayed wood that is also white but may have a lesser degree of delignification (Agosin et al. 1990). This is a type of selective white rot that causes extensive delignification in *Nothofagus* wood. Many different fungi have been suggested to be associated with this decay including *Ganoderma applanatum*, *Ganoderma australe, Armillariella limonea,* and *Phlebia chrysocrea* (Knoche et al. 1929; Kühlwein 1963; González 1980; Zadrazil et al. 1982; Philippi 1983; Ramírez and González 1985; Dill and Kraepelin 1986; González et al. 1986; 1989; Peredo 1987; Eyzaguirre 1988; Ramírez 1988; Agosin et al. 1990; Ferraz et al. 2000, 2001; Elissetche et al. 2001; Elissetche et al. 2007). Other studies have focused on the identification of species and evaluation of their ligninolytic enzyme activities. These studies included *G. applanatum, G. australe, Bjerkandera adusta, Anthracophyllum discolor, Trametes versicolor,* and *Stereum hirsutum* (Silva et al. 1990; Garnica et al. 1997; Garnica and Valenzuela 1998; Arias et al. 1999; Parada et al. 2000; Palma et al. 2005; Donoso et al. 2008; Guillen 2008; Guillen et al. 2008; Oses et al. 2008; Tortella et al. 2008; Acevedo et al. 2011; Taboada-Puig et al. 2011). All of these fungi were identified using traditional methods of macroscopic and microscopic observations and few collections have been deposited in herbaria.

The previous reports demonstrate the large interest in white-rot fungi that may be found in Chile; but information on the diversity of fungi is far from complete. In addition, identification using DNA sequencing has not been performed. This report shows results of an initiative to identify white-rot fungi in a very unique area of Chile, the Chiloé National Park in the Los Lagos Region of Chile and provides new knowledge of the white rot fungi, identified by sequencing the complete ITS rDNA or partial LSU

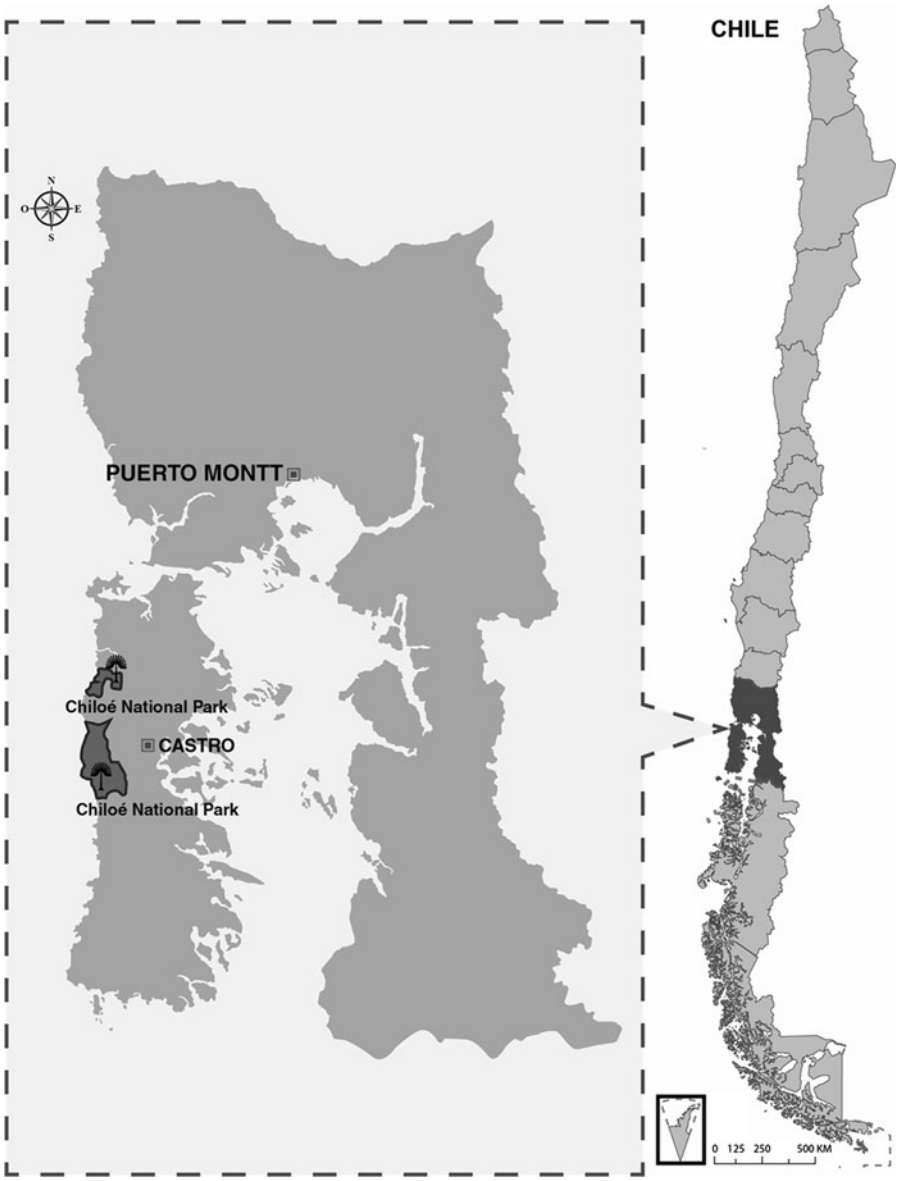

Fig. 1 Los Lagos Region–Chiloé National Park in Chile where samples for this study were obtained

rDNA, as well as the characterization of the type of white rot associated with them.

Materials and methods

Collection and isolation of rot fungi

Collection location and methodology

The samples were collected in the forests of Chiloé National Park, located in Chiloé Archipelago between latitudes 41° and 43° South (Fig. 1). The collection protocol established fixed-size plots sampling along two trails: El Tepual and De Chile, according to the modified protocol suggested by Mueller et al. (2004). Each fixed-size plot sampling was determined as a plot of a single transect, setting circular sub-plots of 5 meters radius every 50 linear meters of transect. Wood of different stages of decay was collected in each sub-plot. A total of 60 samples of wood were collected and placed in sterile plastic bags and taken to the laboratory where they were stored at 4 °C for a month before inoculation into the culture medium. The

samples of wood were divided into 4 stages of decay according to González et al. (1989): I (initial), M (medium), A (Advanced) and F (final).

Inoculation in culture medium

Wood segments of 0.5 square cm were aseptically cut from the collected samples and placed in Petri plates containing a selective medium for basidiomycetous fungi. Plates were incubated at room temperature at approximately 24 ± 2 °C. The culture medium was prepared with 15 g Difco-agar, 15 g Bacto-malt extract, 2 g yeast extract and 0.06 g benlate (methyl-1-(butylcarbamoyl)-2-benzimidazole-carbamate).
The obtained suspension was sterilized at 121 °C for 25 min. Once cooled to 45 °C, 0.01 g streptomycin sulfate and 2 ml of lactic acid were added. The isolation of basidiomycetous fungi was confirmed by microscopic observation of septate hyphae and presence of clamp connections in some cultures (Silva et al. 1990).

Identification of white-rot fungi

To identify the white-rot basidiomycetes, a culture method was used to determine the presence of polyphenol oxidases; these formed a brownish circle around the growing mycelia in the culture medium, according to Bavendamm (1928). Although a few white rot fungi have been previously reported to not produce a reaction using the Bavendamm test, this culture assay was used as an initial screening procedure. If a negative reaction was found, the fungus was still sequenced to determine its identity.

Identification of fungi

The liquid medium for obtaining dry mycelium was prepared with 10 g Bacto-malt extract and sterilised as mentioned previously. Erlenmeyer flasks 500 ml containing 125 ml were inoculated with the mycelium to be identified and incubated at room temperature in a shaker at 150 rpm for a week. The mycelium produced was filtered and washed according to the protocol described by Montiel (2005). After washing, the mycelium was dried in an oven at 45 °C for 12 h. DNA extraction was carried out using the protocol of Cubero et al. (1999). Integrity of extracted DNA was determined by gel electrophoresis. DNA amplification was performed through PCR (Biorad Thermal Cycler); the complete ITS rDNA or partial LSU rDNA were amplified using fungal specific primers ITS1F and ITS4 or LROR and LR5 respectively. The primers LROR and LR5 were used only when the BLAST, with ITS sequence could not differentiate at the species level.

The PCR reaction mix was prepared with 100 ng of genomic DNA, 1×Paq5000 Reaction Buffer (Stratagene), 0.8 mM of dNTPs mix (0.2 mM of each dNTP), 2.5 U Paq5000 DNA polymerase (Stratagene), 0.2 µM of forward primer, 0.2 µM of reverse primer and Milli-Q H_2O to complete a final volume of 50 µl. The PCR reaction consisted of an initial denaturation at 95 °C for 2 min, 30 cycles of amplification (denaturation at 95 °C for 20 s, alignment at 60 °C for 20 s, and an extension at 72 °C for 30 s) and a final extension at 72 °C for 5 min. The fungus from phylum Ascomycota, *Candida dubliniensis* CD36 ATCC, provided by the Oral Biochemistry and Biology Laboratory from Universidad de Chile, was used as a positive control of the reaction. PCR products, prior performing the sequencing reactions, were purified by using the E.Z.N.A. ® Cycle-Pure Kit commercial kit (Omega-Biotech).

Scanning electron microscopy and wood species identification

Wood samples were prepared for scanning electron microscopy (SEM) using techniques described previously by Blanchette and Simpson (1992). Observations were made and photographs taken using a Scanning Electron Microscope Carl Zeiss, model EVO-MA10. The wood species identification was made, using keys of wood anatomy, as reported by Diaz Vaz (1979).

Results and discussion

The collected wood samples for this study belonged to the following hardwood tree species (Table 1): arrayán (*Luma apiculata*), coihue (*Nothofagus dombeyi*), canelo (*Drimys winteri*) and ulmo (Eucryphia cordifiola). However, for P.CH-1, P, CH-11 and P.CH-16, because the woods were in a state of advanced decay, identification of the wood was not possible.

A total of 14 pure cultures of white-rot fungi were selected by Bavendamm (1928) test. Table 1 shows

Table 1 Fungal taxa identified from wood samples with comparisons (% query cover and % identity) to the best BLASTn match with the NCBI GenBank database

Code	Primers for ITS or LSU	Mycobank classification	Best blast match	Percentage query cover	Percentage identity	Wood Identification	Stage of decay	Type of decay	References to Chile	GenBank accession number
P.CH-1	LROR-LR5	*Polyporales* *Meruliaceae* *Phlebia*	*P. chrysocreas* AY586695.1	100	97	Unidentified	Advanced	Selective	Barrasa et al. (1992); Schmidt (2006); Peredo (1987)	KF562007
P.CH-2	LROR-LR5	*Polyporales* *Meruliaceae* *Mycoacia*	*M. fuscoatra* JN649352.1	100	99	Coigue *N. dombeyi* *Nothofagaceae*	Medium	Simultaneous	None	KF562008
P.CH-4	ITS1-ITS4	*Hymenochaetales* *Tubulicrinaceae* *Hyphodontia*	*H. radula* GQ411525.1	99	99	Canelo *D. winteri* *Winteraceae*	Medium	Simultaneous	None	KF562013
P.CH-8	ITS1-ITS4	*Polyporales* *Meruliaceae Bjerkandera*	*B. adusta* JF439464.1	100	99	Ulmo *Eucryphia cordifolia* *Cunoniaceae*	Medium	Selective	Mujica et al. (1980); Lazo (2001); Minter and Peredo (2006); Guillen (2008)	KF562014
P.CH-9	LROR-LR5	*Polyporales* *Meruliaceae* *Mycoacia*	*M. fuscoatra* JN649352.1	100	99	Coigue *N. dombeyi* *Nothofagaceae*	Medium	Simultaneous	None	KF562009
P.CH-11	LROR-LR5	*Polyporales* *Meruliaceae* *Phlebia*	*P. radiata* AF287885.2	100	99	Unidentified	Advanced	Selective	None	KF562010
P.CH-12	LROR-LR5	*Polyporales* *Phanerochaetaceae* *Phanerochaete*	*P. affinis* EU118652.1	100	97	Canelo *D. winteri* *Winteraceae*	Medium	Selective	None	KF562011
P.CH-13	ITS1-ITS4	*Russulales* *Peniophoraceae Peniophora*	*P. cinerea* GU062269.1	100	99	Ulmo *Eucryphia cordifolia* *Cunoniaceae*	Medium	Selective	None	KF562015
P.CH-15	ITS1-ITS4	*Russulales* *Stereaceae* *Stereum*	*S. gausapatum* FN539048.1	100	100	Canelo *D. winteri* *Winteraceae*	Medium	Simultaneous	None	KF562016
P.CH-16	ITS1-ITS4	*Polyporales* *Phanerochaetaceae* *Phanerochaete*	*P. sordida* FJ228210.1	99	99	Unidentified	Advanced	Selective	None	KF562017

Table 1 continued

Code	Primers for ITS or LSU	Mycobank classification	Best blast match	Percentage query cover	Percentage identity	Wood Identification	Stage of decay	Type of decay	References to Chile	GenBank accession number
P.CH-17	ITS1-ITS4	*Polyporales* *Polyporaceae* *Trametes*	*T. versicolor* JN164965.1	100	99	Coigue *N. dombeyi* *Nothofagaceae*	Medium	Simultaneous	Mujica et al. (1980); Butin and Peredo (1986); Gamundi and Horak (1993); Parada et al. (2000); Lazo (2001); Minter and Peredo (2006); Guillen (2008); Donoso et al. (2008); Tortella et al. (2008)	KF562018
P.CH-19	ITS1-ITS4	*Polyporales* *Meruliaceae* *Bjerkandera*	*B. adusta* JF439464.1	100	99	Coigue *N. dombeyi* *Nothofagaceae*	Medium	Selective	Mujica et al. (1980); Lazo (2001); Minter and Peredo (2006); Guillen, (2008)	KF562019
P.CH-20	ITS1-ITS4	*Polyporales* *Meruliaceae* *Ceriporiopsis*	*C. subvermispora* FJ713106.1	100	99	Canelo *D. winteri* *Winteraceae*	Medium	Selective	None	KF562020
P.CH-22	LROR-LR5	*Polyporales* *Meruliaceae* *Phlebia*	*P. setulosa* GU461313.1	100	97	Arrayan *Luma apiculata* *Myrtaceae*	Medium	Selective	None	KF562012

Classification of the fungus, the wood it was associated with, stage (advanced or moderate) of decay and type of decay (selective delignification or simultaneous attack of all cell wall components) is presented. Reference to previous citation of being found in Chile is also noted

the results obtained from the sequencing reactions. When the nucleotide sequences were subjected to alignment by National Center for Biotechnology Information, GenBank (BLAST) (2013), 12 different species of white rot fungi were identified, of the phylum Basidiomycota, class Agaricomycetes, distributed in 3 orders, 6 families and 9 genera. All pure cultures had a BLAST match of 97 % or greater to species designation. Primers ITS1F and ITS4 identified the following species: *Hyphodontia radula* (P.CH-4), *B. adusta* (P.CH-8 and P.CH-19), *Peniophora cinerea* (P.CH-13), *Stereum gausapatum* (P.CH-15), *Phanerochaete sordida* (P.CH-16), *T. versicolor* (P.CH-17) and *Ceriporiopsis subvermispora* (P.CH-20). However, for some isolates, the BLAST match with ITS1F and ITS4, did not differentiate at the species level since there was a limited number of sequences in the database. To obtain a more precise identification, the primers LROR and LR5 were used. With these primers other isolates were identified as: *Phlebia chrysocreas* (P.CH-1), *Mycoacia fuscoatra* (P.CH-2; P.CH-9), *Phlebia radiata* (P.CH-11), *Phanerochaete affinis* (P.CH-12) and *Phlebia setulosa* (P.CH-22). According to Silva et al. (1990), the Bavendamm test does not give a positive reaction to all white rot fungi but is useful to provide an initial screening method to separate most white rot and brown rot fungi. Silva et al. (1990) add that there are white rot fungi that do not produce detectable polyphenol oxidases in culure, for example *Phanerochaete chrysosporium*. For this study cultures that did not give a positive reaction to the Bavendam test were identified, with a BLAST match of 97 % or greater, as *Sistotrema brinkmanni*. This fungus is difficult to classify and has previously been reported as a brown rot fungus (Wang and Zabel 1990; Ginns and Lefebvre 1993; Lamar et al. 1999). However, some *Sistrotrema* have been found to cause a white rot (Ryvarden and Gilbertson 1994). Recent taxonomic studies have indicated that *Sistotrema* is a polyphyletic assemblage and taxon previously identified as *Sistrotrema brinkmannii* are actually a complex of biological species (Moncalvo et al. 2006). Since more work is needed to determine the phylogenectic relationships of this group and its capacity to cause wood decay is uncertain, it has not been included in this study of white rot fungi.

Fruiting bodies of *C. subvermispora* are often difficult to find in forests and this is likely to be the reason this fungus had not been previously reported from natural forests in Chile. In addition to *C. subvermispora*, other fungi found for the first time in Chile include *H. radula, P. radiata, P. affinis, P. cinerea, S. gausapatum, P. sordida and P. setulosa*.

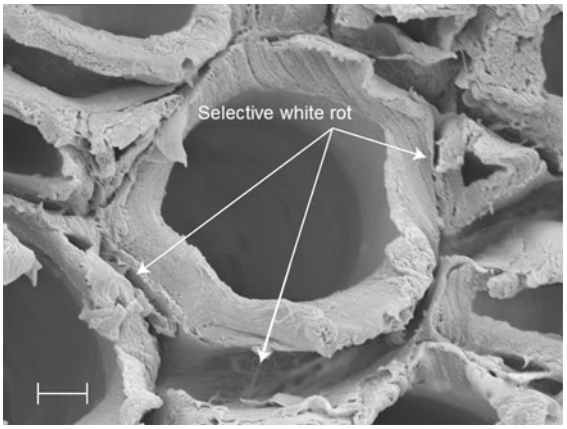

Fig. 2 Transverse section of *D. winteri* decayed by *Ceriporioposis subvermispora* (PCH-20). Selective white rot showing detached cells due to delignification and removal of the middle lamella. *Bar* 5 μm

Agosin et al. (1990) mentioned that the unique environmental factors in the South of Chile, as well as wood species with high syringyl lignin, are seemingly ideal for the growth of white rot fungi that cause a selective delignification of wood. In addition, Dill and Kraepelin (1986) suggest that low temperatures, high humidity and microaerobic conditions may influence positively the processes of wood delignification. The conditions present in Chiloe National Park have rainfall of 1,900 mm a year and an average annual temperature of 10 °C (Meteorological Directorate of Chile, 2011). According to these previous research investigations, these conditions appear ideal for the development of delignification. In our study, the type of white rot was also identified for all samples by scanning electron microscopy (Table 1). Selective delignification was found for nine of the white rots collected (Fig. 2), and five of the collections had decay characteristics of simultaneous white rot (Fig. 3) (Blanchette 1991).

According to Guillen (2008), isolation and characterization of new white-rot fungi is exceedingly important because of their potential biotechnological possibilities. These authors add that there have been few studies completed in Chile on the diversity of wood-rotting fungi. Lazo (1995) indicated that the

Fig. 3 Transverse section of *D. winteri* decayed by *S. gausapatum* (PCH-15). Simultaneous white rot where cell walls were eroded. All cell wall components were degraded resulting in thinned cell walls and areas of localized attack causing holes to form in cell walls. *Bar* 10 μm

richest area of fungal diversity is likely the South of Chile since there is abundant rainfall and many different types of hardwood tree species that occur only in this region of the world. The research reported here demonstrates that a considerable number of diverse white rot fungi were found within a relatively small area of the park. Additional study is needed to obtain more information on all the different fungi involved in wood decay, including the brown rot fungi.

Raberg et al. (2005) suggest it is difficult if not impossible to identify to species level when only the cultural characteristics of basidiomycete mycelium are observed. Prewiit et al. (2008) also adds that these traditional methods are difficult to carry out and identification from just morphological characteristics may lead to wrong conclusions (Moreth and Schmidt 2000; Kim et al. 2005). According to Blanchette et al. (2005), improvements in molecular techniques have provided new tools for identifying microorganisms in wood. Particularly, DNA sequence analysis has been successfully implemented for identifying microorganisms associated with degradation of wood products in service (Schmidt and Moreth 1999; Schmidt and Moreth 2003; Kim et al. 2005; Lim et al. 2005) and microbial diversity in forest ecosystems (Vasiliauskas and Stenlid 1998; Vasiliauskas et al. 2005; O'Brien et al. 2005; Gelsomino et al. 2011). Variability in ITS regions among species appear most useful for taxonomic purposes and has become the region used for barcoding fungi (Begerow et al. 2010; Schoch et al. 2012; Suwannasai et al. 2013). Although not all cultures were identified to species using ITS1F and ITS4, these primers gave good information on the identification of most of the fungi obtained. The use of primers LROR and LR5, belonging to the large subunit, was used to further differentiate some of the isolates that did not provide a good species blast match using ITS1F and ITS4.

The results of this study provide new information about white rot fungi from the temperate rain forests of Chile and provide several new isolates native to Chile for studies involving their degradative ability and potential for use in biotechnological processes.

Conclusions

A total of 14 pure cultures were isolated from the collected samples and were identified as 12 different species of white rot fungi of the phylum Basidiomycota. The cultures had a BLAST match of 97 % or greater to sequences in the GenBank database and were identified as *P. chrysocreas*, *M. fuscoatra*, *H. radula*, *B. adusta*, *P. radiata*, *P. affinis*, *P. cinerea, S. gausapatum*, *P. sordida*, *T. versicolor*, *C. subvermispora* and *P. setulosa*. *C. subvermispora*, *H. radula, P. radiata, P. affinis, P. cinerea, S. gausapatum, P. sordida* and *P. setulosa* are reported from Chile for the first time.

Future work is needed to evaluate the ligninolytic capabilities of many of these isolates since several were selective delignifying fungi and could have potential application for biotechnological uses.

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