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FUNGAL MICROBIOLOGY

Investigations of Biodeterioration by Fungi in Historic Wooden Churches of Chiloé, Chile

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Abstract The use of wood in construction has had a long history and Chile has a rich cultural heritage of using native woods for building churches and other important structures. In 2000, UNESCO designated a number of the historic churches of Chiloé, built entirely of native woods, as World Heritage Sites. These unique churches were built in the late 1700 s and throughout the 1800 s, and because of their age and exposure to the environment, they have been found to have serious deterioration problems. Efforts are underway to better understand these decay processes and to carryout conservation efforts for the long-term preservation of these important structures. This study characterized the types of degradation taking place and identified the wood decay fungi obtained from eight historic churches in Chiloé, seven of them designated as UNESCO World Heritage sites. Micromorphological observations identified white, brown and soft rot in the structural woods and isolations provided pure cultures of fungi that were identified by sequencing of the internal transcribed region of rDNA. Twenty-nine Basidiomycota and 18 Ascomycota were found. These diverse groups of fungi represent several genera and species not previously reported from Chile and demonstrates a varied microflora is causing decay in these historic buildings.

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Introduction

Wood has had a long history of use as construction material on the island of Chiloé, Chile where many extraordinary churches were built with native timbers from the region [1–3]. These structures (Fig. 1) have become important cultural monuments that provide historical information on their unique architecture developed through the integration of European and indigenous cultures which is called the Chilota School of Ecclesiastical Wooden Architecture [4]. From 60 churches of the Chiloé Archipelago, 16 were designated National Historical Monuments by the government of Chile [4] and were declared by United Nations Educational, Scientific and Cultural Organization (UNESCO) as World Heritage sites [4, 5].

Wood placed into the environment, especially wood in the temperate rain forests of southern Chile, is subjected to conditions that are conducive to decay. These conditions include high humidity, excessive rainfall and moderate temperatures throughout the year [6–10]. Although many of the native woods used have natural durability [11, 12], over time, even the most durable woods can succumb to attack by some species of wood-destroying fungi [13]. Among the many different microorganisms that can colonize wood, the fungi that cause white, brown, and soft rot are the predominant agents that destroy wood [8, 14]. The protection of wood used in construction from decay by means of the design is a relatively recent concept not well considered in older buildings [15, 16]. In the case of the Chiloé churches, builders used

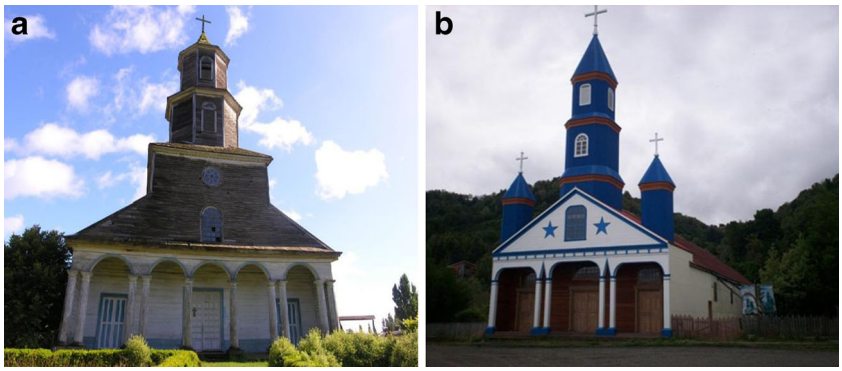

Fig. 1 Wooden churches of Chiloé, Chile showing the structures before restoration (**a**) and after (**b**). Nuestra Señora de Gracia de Nercón Church located in Nercón (**a**) and Nuestra Señora del Patrocinio Church in Tenaún (**b**)

wood considered as very durable, alerce (*Fitzroya cupressoides*) and cypress of the Guaitecas (*Pilgerodendron uviferum*) as well as other woods that were moderately durable, such as coigüe (*Nothofagus dombeyi*), ulmo (*Eucryphia cordifolia*), and canelo (*Drimys winteri*) [2], likely helped preserve the structures for long periods. However, biodeterioration is now a significant problem [2, 3].

A number of studies around the world have documented wood decay problems in important historical wooden structures and archeological woods [13, 17–22]. Although these provided new information to characterize the nature of the attack and processes of degradation, there are few reports providing information on the identification of fungi involved in the decay of historical structures [19]. This has been primarily due to the difficulty in identifying wood decay fungi in culture. Molecular methods, now available to identify fungi in culture by sequencing conserved regions of rDNA, can facilitate identification and provide information on the diverse fungi causing the degradation.

In Chile, investigations of decay in historical buildings and in archaeological wood are very limited [22]. However, some studies have focused on decomposition processes of woods in natural forests [6, 7, 23–38]. These studies and others suggest that there are more than 3,300 species of fungi occurring in Chile and provide a baseline of information on fungal biodiversity for the country [10, 39, 40].

The objectives of this study were to characterize the type of degradation found in eight historic wooden churches in Chiloé, Chile and identify the diversity of wood decay fungi causing the decay by sequencing the ITS region of rDNA.

Materials and Methods

Collection of Wood and Isolation of Fungi

One hundred and fifty eight samples of wood in different stages of decay were collected from eight historical churches in Chiloé, Chile (Table 1). Small segments of wood were taken from different timbers throughout all locations of the churches that appeared decayed. The churches, designated as UNESCO World Heritage sites [41], were located in the villages of Nercón, Castro, Rilán, Achao, Quinchao, San Juan and Tenaún and one other historically significant church located in Calen was included in these studies (Fig. 2). The samples of decayed wood were placed in sterile plastic bags and kept cool while transported to the Biodeterioration Laboratory at the Wood Engineering Department of the Universidad del Bío-Bío, Biodeterioration and Biodegradation of Materials Laboratory at the School of Civil Construction of the Universidad of Valparaíso in Chile, or the Forest and Wood Microbiology Laboratory at the University of Minnesota, St. Paul, MN, USA and maintained at 4 °C until cultured.

Different cultural media were used including malt extract agar (15 g malt extract, 15 g agar), acidified malt extract agar (15 g malt extract, 15 g agar and 2 ml concentrated lactic acid added after autoclaving) and a selective culture medium for isolating basidiomycetes (15 g malt extract, 15 g agar, 2 g yeast extract, 0.06 g benlate (Methyl-L-(butylcarbamoil)-2-bencimidazol-carbamate), 2 ml concentrated lactic acid, and 0.01 g streptomycin sulfate added after autoclaving).

Small segments of the decayed wood samples were cut and aseptically placed in Petri dishes of each of the culture media mentioned above. Incubation was at room temperature (24± 2 °C) to allow for the development of fungi, and then subcultures were made to obtain pure fungal cultures.

Table 1 Churches in Chiloé, Chile where investigations were done

Church	Village	UNESCO World Heritage site
Nuestra Señora de Gracia de Nercón	Nercón	Yes
San Francisco	Castro	Yes
Santa María de Loreto de Rilán	Rilán	Yes
Santa María de Loreto	Achao	Yes
Nuestra Señora de Gracia	Quinchao	Yes
San Juan Bautista de San Juan de Coquihuil	San Juan	Yes
Iglesia de Nuestra Señora del Patrocinio	Tenaún	Yes
Calen	Calen	No

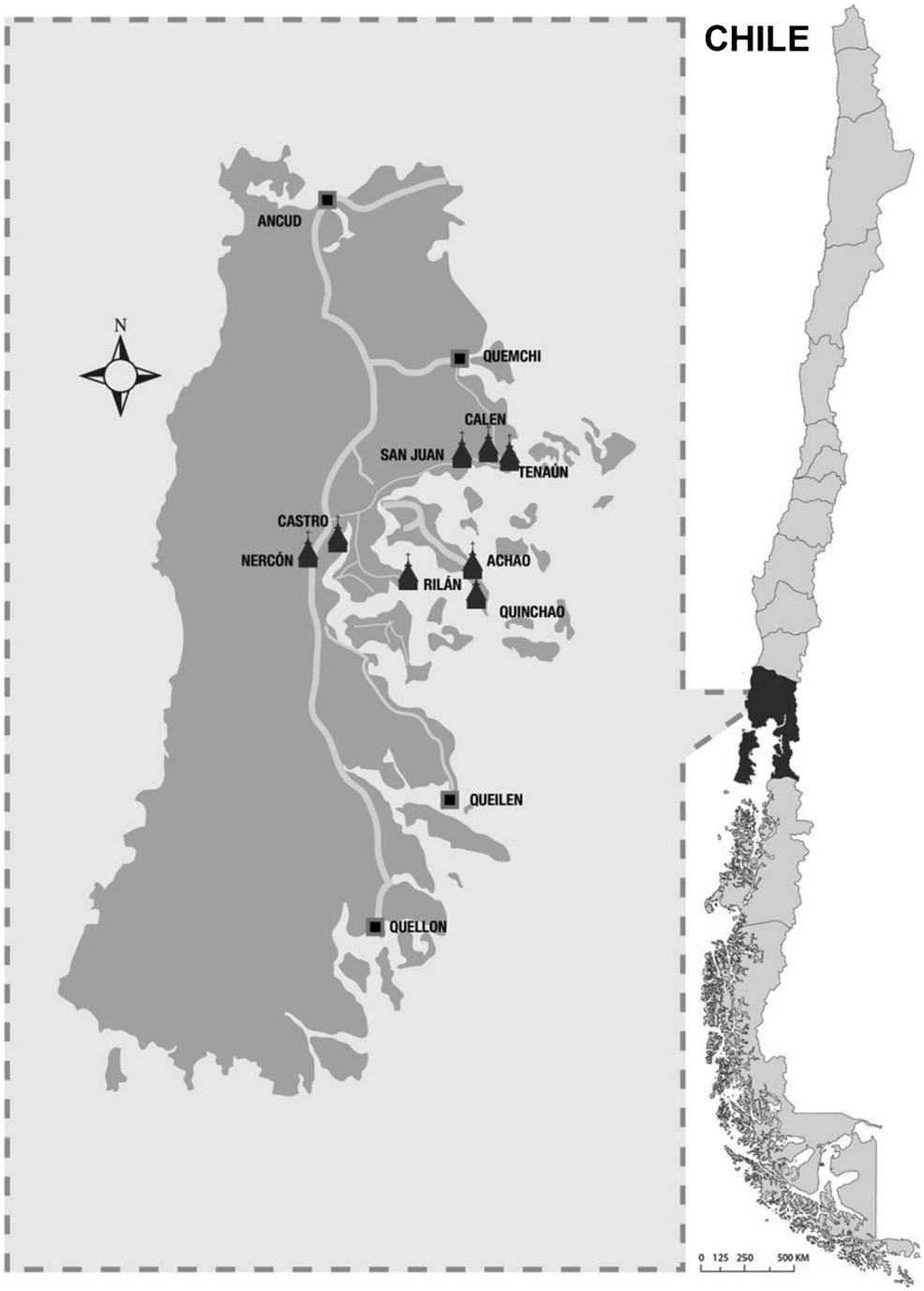

Fig. 2 Location of the Chiloé churches where decay investigations were completed

Identification of Fungi from the Wood

A liquid medium, used for growing pure isolates of cultured fungi and to obtain dry mycelium for molecular analyses, was prepared with 10 g Bacto-malt extract per liter of distilled water and sterilized at 121 °C for 25 min. Approximately 125 ml of the liquid was placed in 500-ml Erlenmeyer flasks which were inoculated with the fungi Mycelium to be identified and incubated at room temperature in a shaker at 150 rpm for 1 week. The mycelium was filtered and washed according to the protocol described by Montiel [42]. After washing, the mycelium was dried in an oven at 45 °C for 12 h. For studies done at the University of Bio-Bio and the University of Valparaíso, DNA extraction was carried out using the previous published protocol of Cubero et al. [43]. Integrity of extracted DNA was determined by gel electrophoresis, formed by 1 % (*w*/*v*) agarose dissolved in a TAE Buffer 0.5X with 5 μL of ethidium bromide (10 mg/mL). Loading buffer was prepared with 1 μL of Gel Loading Dye 6X for every 5 μL of DNA solution. The electrophoretic run was performed at 90 V for

40 min. DNA observation was done by using a UV transilluminator. DNA amplification was performed using PCR in a Biorad Thermal Cycler. Complete ITS rDNA were amplified using fungal specific primers ITS1F and ITS4. The PCR reaction mix was prepared with 100 ng of genomic DNA, 1X Paq5000 reaction buffer, 0.8 mM of dNTPs mix (0.2 mM of each dNTP), 2.5 U Paq5000 DNA polymerase, 0.2 μM of forward primer, 0.2 μM of reverse primer and MQ H_2O to complete a final volume of 50 μL. The PCR reaction considered an initial denaturation at 95 °C for 2 min, 30 cycles of amplification, and a final extension at 72 °C for 5 min. Each amplification cycle considered a denaturation at 95 °C for 20 s, alignment at 60 °C for 20 s, and an extension at 72 °C for 30 s. 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 to performing the sequencing reactions, were purified by using the E.Z.N.A. ® Cycle-Pure Kit commercial kit (Omega-Biotech). For fungi isolated at the University of Minnesota, methods used were as previously described by Blanchette et al. [19]. These investigations were done to document the diversity of wood decay fungi found in these structures and not to provide a quantitative assessment of where each fungus was located within each church.

Scanning Electron Microscopy and Wood Species Identification

Wood samples were prepared for scanning electron microscopy (SEM) using techniques described previously by Blanchette and Simpson [44]. 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 [45].

Results

The collected wood samples were comprised of the following tree species: alerce (*Fitzroya cupressoides*), cypress of the Guaitecas (*Pilgerodendron uviferum*), coigüe (*Nothofagus dombeyi*), ulmo (*Eucryphia cordifolia*), and canelo (*Drimys winteri*). Micromorphological characterization of the wood samples indicated that different types of decay and various stages of decay were present. Brown rot and white rot were evident in many beams and timbers in all of the churches; however, these different types of decay were found in different timbers. Decay was often extensive with advanced stages of degradation present. Some soft rot was also evident in exterior woods that were made from the decay-resistant alerce and cypress (Fig. 3).

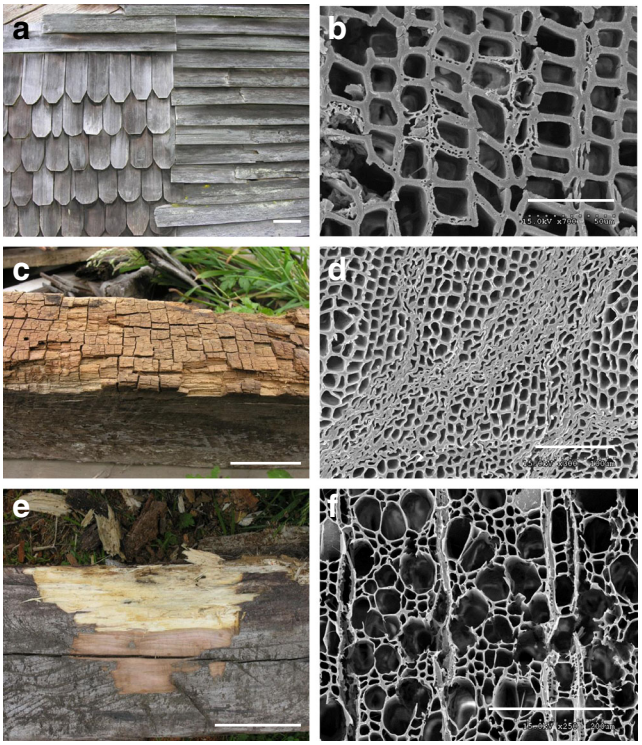

Fig. 3 Types of wood decay found in the churches of Chiloé, Chile. **a** Exterior woods with soft rot. **b** Scanning electron micrograph of transverse section of a wood showing soft rot consisting of cavities within the secondary wall. **c** Brown rot in timber removed during restoration. **d** Scanning electron micrograph of transverse section showing tracheids with brown rot. Depolymerization of cellulose caused a loss of cell wall integrity resulting in distorted and collapsed cells. **e** White rot in timber removed during restoration. **f** Scanning electron micrograph of transverse section of wood with a simultaneous white rot where all cell wall layers were eroded. Bar=15 cm (**a**), 50 μm (**b**), 10 cm (**c**, **e**), 100 μm (**d**), and 200 μm (**f**).

Since the objectives of this study were to identify the wood decay fungi present in each church, only one isolate of each taxa identified is presented for each church. A total of 47 pure cultures of different fungal taxa were obtained from the sampling, of which 7 were isolated from wood samples from the Achao church, 3 from the Castro church, 10 from the Calen church, 9 from the Quinchao church, 3 from the Nercón church, 7 from the San Juan church, 6 from the Tenaún church, and 2 from the Rilán church. Some samples of decayed wood, especially those with advanced stages, did not yield wood-destroying fungi.

Results obtained from sequencing reactions of the rDNA identified 29 pure cultures of the phylum Basidiomycota belonging to 8 orders and several that were not determined since only a broad taxon category was possible (Table 2), as well as 18 pure cultures of Ascomycota belonging to 5 orders and several of these matched only broad categories of fungi (Table 3). Of all pure cultures identified, three cultures, Castro 20, Tenaún 71A, and Rilán 17-1, matched only sequences from environmental samples that were uncultured fungi. Also, there were three cultures that could be identified only very broadly as members

Table 2 Basidiomycota identified from wood samples with comparisons (% query cover and % identity) to the best BLAST match with the NCBI GenBank database

Culture	Best BLAST match	Order	Query coverage	Max identity	GenBank accession #
Achao 41	*Hyphodermella corrugata*	Polyporales	98	100	KF638510
Achao 44	*Gymnopus subpruinosus*	Agaricales	100	100	KF638511
Achao 46	*Laetiporus sulphureus*[a]	Polyporales	93	82	KC514825
Achao 47	*Stereum hirsutum*	Russulales	99	98	KF638513
Achao 48	*Bjerkandera adusta*	Polyporales	98	99	KF638514
Achao 50	*Fomitiporella caryophylli*[a]	Hymenochaetales	96	94	KF638515
Castro 19-4	*Hyphodermella corrugata*[a]	Polyporales	97	93	KF638516
Castro 20-1	*Clitopilus hobsonii*[a]	Agaricales	100	95	KF638517
Calen 59	*Ceriporiopsis* sp.	Polyporales	90	99	KF638518
Calen 60-2	*Peniophorella* sp. NH-2006a	Hymenochaetales	100	99	KF638519
Calen 62	*Stereum hirsutum*	Russulales	99	99	KF638520
Calen 62-2	*Trametes versicolor*	Polyporales	100	100	KF638522
Calen 65	*Postia dissecta*	Polyporales	95	100	KF638524
Quinchao 21	*Dacrymyces stillatus*	Dacrymycetales	87	99	KF638525
Quinchao 22A	*Ceriporiopsis* sp. LF219	Polyporales	73	98	KF638526
Quinchao 25B	*Sistotrema brinkmannii*	Cantharellales	100	99	KC514823
Quinchao 25-2 CH	*Bjerkandera adusta*	Polyporales	100	99	KF638527
Quinchao 26A	*Galzinia* sp.[a]	Corticiales	66	93	KF638528
Quinchao 26-5 CH	*Entoloma tenellum*[a]	Agaricales	42	94	KF638529
Quinchao 32	*Phlebia rufa*	Polyporales	98	99	KF638531
Nercón 12B	*Dacrymyces stillatus*	Dacrymycetales	98	99	KF638533
San Juan 51B	*Trametes versicolor*	Polyporales	98	99	KF638535
San Juan 53	*Coniophora puteana*	Boletales	100	100	KF638536
San Juan 54-1 CH	*Phlebia rufa*	Polyporales	100	99	KF638537
Tenaún 71B	*Trametes versicolor*	Polyporales	100	100	KF638540
Tenaún 71-1 CH	*Sistotrema brinkmanni*[a]	Cantharellales	96	92	KF638541
Tenaún 73	*Aurantiopileus mayanensis*	Polyporales	98	99	KF638544
Tenaún 74	*Peniophorella* sp.	Hymenochaetales	93	99	KF638545
Tenaún 91	*Laetiporus sulphureus*[a]	Polyporales	78	83	KC514814

[a] BLAST match is 95 % or less and the correct taxon designation of the isolate is unknown

of the phylum Ascomycota (Calen 61A, Nercón 6-4 CH, and San Juan 50-1 CH).

For the following cultures identified at the generic level, Calen 59, Quinchao 22A (*Ceriporiopsis* sp.), Calen 60-2, Tenaún 74 (*Peniophorella* sp.), Quinchao 26A (*Galzinia* sp.), Calen 61-3CH (*Penicillium* sp.), Nercón 9-1CH (*Alternaria* sp.), Tenaún 71A (*Trichoderma* sp.), and Rilan 15-1 (*Verticillium* sp.), these sequences did not match any deposited sequences to species. Fungi of the phylum Basidiomycota that had a best BLAST match of 97 % or greater to sequences in the Genbank database included *Hyphodermella corrugate* (Achao 41), *Gymnopus subpruinosus* (Achao 44), *Stereum hirsutum* (Achao 47; Calen-62), *Bjerkandera adusta* (Achao-48; Quinchao 25-2 CH), *Trametes versicolor* (Calen 62-2; San Juan 51B, Tenaun 71B), *Postia dissecta* (Calen 65), *Dacrymyces stillatus* (Quinchao-21; Nercón 12B), *Sistotrema brinkmannii* (Quinchao 25B), *Phlebia rufa* (Quinchao 32; San Juan 54-1 CH), *Coniophora puteana* (San Juan 53), and *Aurantiopileus mayanensis* (Tenaún 73). Also, isolates that only matched fungi in the Ascomycota with a BLAST match of 97 % or greater in the Genbank database included *Endothia viridistroma* (Achao 45), *Penicillium commune* (Calen 58-2 CH), *Alternaria frumenti* (Calen 60-3 CH), *Pochonia bulbillosa* (Calen 61; San Juan 51A), *Scleroconidioma sphagnicola* (Quinchao 23), *Scytalidium lignicola* (Quinchao 33A), and *Tricladium terrestre* (San Juan 52-2 CH). The majority of the cultures showed a high BLAST match. However, the cultures Achao 46 (*Laetiporus sulphureus*), Achao 50 (*Fomitiporella caryophylli*), Castro 19-4 (*Hyphodermella corrugata*), Castro 20-1 (*Clitopilus hobsonii*), Quinchao 26-5CH (*Entoloma tenellum*), Tenaún 71-1CH (*Sistotrema brinkmannii*), Tenaún 91 (*Laetiporus sulphureus*), and San Juan 55-2CH (*Dendryphion penicillatum*) had identities from the best BLAST match ranging from 82 to 95 %, and the correct identity remains unknown.

Table 3 Ascomycota identified from wood samples with comparisons (% query cover and % identity) to the best BLAST match with the NCBI GenBank database

Culture	Best BLAST match	Order	Query coverage	Max identity	GenBank accession #
Achao 45	*Endothia viridistroma*	Diaphortales	100	99	KF638546
Castro 20	Uncultured *Pochonia*[a]	Hypocreales	100	95	KF638547
Calen 58-2 CH	*Penicillium commune*	Eurotiales	100	99	KF638548
Calen 60-3 CH	*Alternaria frumenti*	Pleosporales	100	100	KF638549
Calen 61	*Pochonia bulbillosa*	Hypocreales	100	99	KF638550
Calen 61A	*Fungal endophyte* sp.	Not determined	95	100	KF638551
Calen 61-3 CH	*Penicillium* sp. 12NJ04	Eurotiales	100	99	KF638552
Quinchao 23	*Scleroconidioma sphagnicola*	Not determined	100	99	KF638553
Quinchao 33A	*Scytalidium lignicola*	Helotiales	94	99	KF638554
Nercón 6-4 CH	Ascomycete sp. Uf-2007a	Not determined	99	97	KF638555
Nercón 9-1 CH	*Alternaria* sp. XAE_154	Pleosporales	99	100	KF638556
Rilan 15-1	Uncultured *Verticillium*	Hypocreales	100	99	KF675189
Rilan 17-1	Uncultured Ascomycete	Not Determined	100	99	KF675190
San Juan 50-1 CH	Ascomycota sp. 2 AJMH-2010	Not determined	100	97	KF638557
San Juan 51A	*Pochonia bulbillosa*	Hypocreales	99	99	KF638558
San Juan 52-2 CH	*Tricladium terrestre*	Helotiales	100	99	KF638559
San Juan 55-2 CH	*Dendryphion penicillatum*[a]	Pleosporales	99	89	KF638538
Tenaún 71A	Uncultured *Trichoderma*	Hypocreales	98	99	KF638560

[a] Blast match is 95 % or less and the correct taxon designation of the isolate is unknown

Discussion

In Chile, there is limited information on the wood-destroying fungi associated with wood in service or on fungi causing degradation in historic wooden buildings. Since many of the wood decay fungi have similar mycelial morphology in culture and do not produce fruiting bodies readily, Moreth and Schmidt [46] indicated that the traditional methods of identification using morphological characteristics is difficult and not always reliable leading to errors. Advances in molecular biological techniques have provided tools to identify the microorganisms in wood with precision. In particular, rDNA sequence analysis has been used successfully to identify the microorganisms associated with the deterioration of structural wood in different regions of the world [12, 21, 47, 48] and microbial diversity in forest ecosystems [49–52]. The results obtained in the studies presented here show that a large number of the decay fungi found in the churches of Chiloé are white rot fungi and only a few of the isolates are known to cause brown rot (*Coniophora, Laetiporus, Postia*). Brown rot fungi are usually the most prevalent organisms causing decay in buildings [8]. Since the samples taken from these historic structures had to be small in size and some of the decayed wood appeared very old, not all samples yielded viable isolates of fungi. In some cases, the decay may have occurred long ago and the fungus was no longer active or had moved into areas of the structure where sampling was not possible. Although, some samples did not yield cultures of fungi, the large number of samples obtained (158 from all of the churches) provided a large, diverse group of wood decay fungi and many of these are known taxa that produce white rot. White rot fungi are known to be more prevalent in hardwoods and they likely represent native fungi that have a preference for colonizing Chilean hardwoods, such as *Nothofagus dombeyi*, *Eucryphia cordifolia*, and *Drimys winteri* that were used in the structures. In addition to the brown and white rot fungi that were found, several ascomycetes with the potential to cause soft rot decay were isolated from other woods such as the exterior boards made from alerce and cypress. A few fungi, such as *Trichderma*, *Alternaria,* and *Verticilium,* are not usually considered wood decay fungi but are listed since they were found associated with the decayed wood. This report also expands our knowledge about the diversity of wood decay fungi found in Chile. The genera *Peniophorella*, along with the species *Sistotrema brinkmannii, Aurantiopileus mayanensis, Endothia viridistroma, Pochonia bulbilosa, Scleroconidioma sphagnicola*, and *Tricladium terrestre* are reported for the first time in Chile. For several fungi, a best BLAST match of 95 % or less was obtained with known sequences in the database. It is likely that some of these fungi represent new species or even different genera than the best BLAST match indicated and phylogenetic analyses using other genes will be necessary to provide better clarification on the identity of these isolates. These fungi could be labeled as 'unknown' but we have selected to identify them to the best match available which gives an indication of their closest taxonomic position that is currently known.

Knowledge of the type of decay present in historic structures and the current condition of the decayed wood provides valuable information needed for conservation efforts. Brown rot fungi are known to cause significant strength losses in wood even during incipient stages of decay and the presence of this type of decay requires remedial action and removal of all affected woods (8). Less strength losses are associated with the early stages of white rot and wood with incipient stages of white rot could be tolerated if the decay process can be stopped by controlling moisture. This study also shows that even the most decay-resistant wood species used in the construction of the churches, such as alerce, may be degraded over time. Soft rot fungi appear to be the major cause of decay in this wood. These fungi are poorly understood but recent findings show that they may also be controlled by excluding moisture (21). New information from this investigation identifies the main decay causing fungi that are active in the historic Chilean churches. This information will be useful in future studies to design experiments that help to elucidate the role of these fungi in the degradation processes of Chilean woods and to determine the best methods for controlling them.

Conclusions

All types of decay, including brown rot, soft rot, and white rot were identified in the Churches and a diverse group of wood decay fungi were found. Twenty-nine Basidiomycota and 18 Ascomycota were isolated and identified. Most rDNA sequences of the ITS region were found to match fungi previously reported; however, some cultures did not have sequences that matched known isolates and additional phylogenetic studies are necessary for further characterization. From the isolates obtained, we found two genera and six species that are reported for the first time in Chile. This study is the first in Chile to identify microorganisms associated with the decay of wood in the historic Chiloé churches and provides a better understanding of the fungal diversity associated with wood decomposition processes in these culturally important structures.

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