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Phylogenetic analysis and discoloration characteristics of major molds inhabiting woods. Part 3. Genus *Cladosporium*

Young Min Lee, Yeongseon Jang, Gyu-Hyeok Kim and Jae-Jin Kim*

Division of Environmental Science and Ecological Engineering, College of Life Sciences and Biotechnology, Korea University, Seoul, Korea

*Corresponding author.
Division of Environmental Science and Ecological Engineering, Korea University, 5-1 Anam-dong, Seongbuk-gu, Seoul, 136-701, Korea
Phone: +82-2-3290-3049
Fax: +82-2-953-0737
E-mail: jae-jinkim@korea.ac.kr

Abstract

The genus *Cladosporium* Link is one of the most well-known dark molds causing sapwood discoloration. Although the *Cladosporium* species are widespread in the world, little is known about the extent to which they stain wood. Twenty-eight isolates from seven different tree species in Korea were obtained and investigated in this study. Nine species, including three unknown *Cladosporium* species, were identified based on their genotypic characteristics. *Cladosporium cladosporioides* was the dominant species, followed by *C. rectoides*, *C. perangustum*, *C. pseudocladosporioides*, *C. tenuissimum*, and *C. ramotenellum*. Their discoloration ability was determined with sapwood blocks of Japanese red pine (*Pinus densiflora*) and radiata pine (*Pinus radiata*). On both blocks, *Cladosporium tenuissimum* and *C. cladosporioides* showed the highest level of discoloration.

Keywords: actin; *Cladosporium*; dark mold; phylogeny; translation elongation factor 1-α; wood discoloration.

Introduction

The genus *Cladosporium* Link is one of the largest genera of hyphomycetes, which currently comprises more than 772 names (Dugan et al. 2004). The *Cladosporium* species are widespread in the world and are found on dead leaves and woody plants. Most species exist as secondary invaders on necrotic leaf spots, and they have frequently been isolated from air, soil, food, paint, textiles, and numerous other substrates (Samson et al. 2000; Schubert 2005). They are also known to be common endophytic fungi (Brown et al. 1998; El-Morsy 2000). *Cladosporium* is one of the most heterogeneous fungi. Numerous, superficially similar genera have been assigned to this genus. Previous definitions or distinctions from other genera were imprecise. In recent years, clear delimitations that were based on morphological and molecular studies have been established (Schubert et al. 2007; Zalar et al. 2007; Bensch et al. 2010). Because single-gene phylogeny did not resolve a phylogenetic structure within the *Cladosporium* species, an analysis of the more variable genes was used to support the species clades.

The genus *Cladosporium* is a well-known dark mold that can cause sapwood discoloration. Similar to *Trichoderma* and *Penicillium*, *Cladosporium* commonly colonize wood surfaces such as conifers, hardwood, round wood, lumber, and wood products (Schmidt 2006). The organisms live on nutrients within sapwood's parenchyma cells. As reported by Butcher (1968) and Wang and Zabel (1990), *Cladosporium* has been isolated from chromate copper arsenate (CCA)-treated wood and discolored wood products. In Korea, Kim et al. (2007) isolated the following four *Cladosporium* species from CCA-treated radiata pine boards: *C. oxysporum*, *C. sphaerospermum*, *C. herbarum*, and *C. cladosporioides*. Because it was reported that the species might play an influential role in the deterioration and the discoloration of CCA-treated radiata pine board, it is important to control these fungi. Also, it was known as a soft-rot fungus (Duncan and Eslyn 1966; Choi 2004; Kim et al. 2007). However, there is little information about the species isolated and the extent to which they stain wood.

In this study, a multilocus DNA sequence analysis was undertaken to establish species identities and to elucidate species diversity within the genus *Cladosporium*. This analysis focused on the following three gene regions: internal transcribed spacer (ITS), actin (ACT), and translation elongation factor 1-α (TEF). The discoloration ability of these species, including their growth patterns and their color on sapwood blocks, was also investigated.

Materials and methods

Fungal isolation and determination of growth rates

In addition to CCA-treated larch and radiata pine and creosote-treated wood, the following wood species were investigated: bamboo, Douglas-fir (*Pseudotsuga menziesii*), Japanese red pine (*Pinus densiflora*), Korean pine (*Pinus koraiensis*), larch (*Larix kaempferi*), radiata pine (*Pinus radiata*), and Siberia spruce (*Picea jezoensis*). These wood samples were mostly obtained from sawmills and log yards in Korea from 2000 to 2007 (Table 1). Fungal isolation and purification were performed as described by Huh et al. (2011). Single-conidial isolates were inoculated on malt extract agar (MEA; malt extract 20 g, agar 15 g, distilled water 1000 ml) and incubated at 25°C for 7 days in darkness.

Table 1 *Cladosporium* isolate identification based on the ITS, ACT, and TEF sequence data and their discoloration rate.

Fungus	Isolate number[a]	Source[b]	Sample location[c]	Discoloration rate[d] RP* 7/14/21/28 days	Discoloration rate[d] JP* 7/14/21/28 days
C. cladosporioides	KUC1384	KP	YJ		
	KUC1385	KP	YJ	0/3/4/4	1/4/5/5
	KUC1420	JPLB	BH		
	KUC1516	KPLB	GP		
	KUC1545	KPLB	GP		
	KUC1580	LA	YJ		
	KUC1699	JP	BH		
	KUC1701	JP	BH	1/4/5/5	2/4/5/5
	KUC3006	CCARP	IC		
	KUC3076	CCALA	SU		
C. perangustum	KUC1462	JPLB	BH	0/2/3/4	2/3/5/5
	KUC1767	RP	BS	0/1/3/4	1/3/5/5
	KUC5085	SS	IC		
C. pseudocladosporioides	KUC1671	KP	GP	0/2/5/5	2/3/5/5
	KUC1700	JP	BH	0/2/4/5	2/4/5/5
	KUC4095	BB	JJ		
C. ramotenellum	KUC3027	CCARP	IC	0/2/4/5	0/3/5/5
C. rectoides	KUC1421	JPLB	BH	0/2/4/4	2/4/5/5
	KUC1515	KPLB	GP		
	KUC1667	KP	GP	0/1/4/5	1/3/5/5
	KUC5009	CTW	GM		
C. tenuissimum	KUC1698	JP	BH	1/3/4/5	2/4/5/5
	KUC1736	RP	IS		
	KUC1903	DF	OY	1/3/4/5	2/4/5/5
Cladosporium sp. 1	KUC1299	JP	BH	0/0/1/2	0/1/2/3
Cladosporium sp. 2	KUC1766	RP	BS	0/1/3/5	1/3/5/5
	KUC1773	RP	BS	0/2/4/5	0/2/4/5
Cladosporium sp. 3	KUC3009	CCARP	IC	0/2/4/5	0/2/4/5

[a]KUC: Korea University Culture Collection, Seoul, Korea. [b]BB, bamboo; CCALA, CCA-treated larch; CCARP, CCA-treated radiata pine; CTW, creosote-treated wood; DF, Douglas-fir; JP, Japanese red pine; JPLB, Japanese red pine lumber; KP, Korean pine; KPLB, Korean pine lumber; LA, larch; RP, Radiata pine; SS, Siberia spruce. [c]BH, Bonghwa; BS, Busan; GP, Gapyeong; GM, Gwangmyeong; IC, Incheon; IS, Iksan; JJ, Jinju; OY, Onyang; SU, Seoul; YJ, Yeoju. [d]0–5; 0, no discoloration; 5, discoloration on entire wood surface in four replicates. *RP, Radiata pine; JP, Japanese red pine.

To determine growth rates, strains were inoculated on potato dextrose agar (PDA; Difco potato dextrose agar 39 g, distilled water 1000 ml), 2% malt extract agar (MEA), and oatmeal agar (OA; Difco oatmeal agar 72.5 g, distilled water 1000 ml). They were then incubated at 25°C in the dark. The colony diameters of the three replicates of each isolate were recorded at 48 h intervals for 14 days. Colony color and size were also studied.

DNA extraction, polymerase chain reaction (PCR), and DNA sequencing

Genomic DNA was extracted according to Huh et al. (2011). The ITS region including the 5.8S ribosomal DNA (rDNA) gene was amplified using the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), as described by White et al. (1990) and Gardes and Bruns (1993). Two other loci were sequenced to obtain additional sequence information. As described by Carbone and Kohn (1999), part of the ACT gene, with primers ACT-512F (5′-ATGTGCAAGGCCGGTTTCGC-3′) and ACT-783R (5′-TACGAGTCCTTCTGGCCCAT-3′), and part of the TEF gene, with primers EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1-986R (5′-TACTTGAAGGAACCCTTACC-3′), were amplified. PCR reactions were performed on a Bio-Rad MyCycler (Bio-Rad, Hercules, CA, USA). The initial denaturation step was performed at 95°C for 7 min, which was then followed by 30 cycles at 95°C (30 s), 51°C (30 s), 72°C (30 s), and a final 7 min extension step at 72°C. The cycling conditions for ACT and TEF genes followed those of Carbone and Kohn (1999) and Crous et al. (2004), respectively. As explained in Huh et al. (2011), amplicons were detected, purified, and sequenced. The sequences obtained in this study were deposited in GenBank under accession numbers JN033458-JN033485, JN033486-JN033512, JN033513-JN033540, respectively for ITS, ACT, and TEF.

Phylogenetic analysis

The ITS, ACT, and TEF sequences used in this study were aligned with those obtained from GenBank by MUSCLE 3.8.31(Edgar 2004).

They were manually edited with MacClade 4.08 (Maddison and Maddison 2005). The best-fit model of nucleotide substitution was selected under the hierarchical likelihood ratio test (hLTR) by the Modeltest v2.3 (Nylander 2004). Bayesian analyses were performed for three genetic loci in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) with Markov Chain Monte Carlo (MCMC) analysis. *Cercospora beticola* strain (GenBank accession numbers AY840527, AY840458, AY840494, respectively for ITS, ACT, and TEF) served as an outgroup according to Bensch et al. (2010). The ITS region has a limited resolution in the *Cladosporium* species; therefore, the results for the ACT and TEF regions were compared for clade stability. Data sets of the ITS, ACT, and TEF genes were separately analyzed, and combined phylogenetic analysis was conducted.

Wood discoloration characteristics

For the discoloration test, sapwood blocks of Japanese red pine and radiata pine were prepared in four replicates. The test was performed according to the procedure described by Huh et al. (2011). The middle of each wood sample was inoculated with each fungal species grown on MEA. The degree of discoloration was measured every 7 days for 4 weeks, and the discoloration characteristics of color and colonizing patterns were also observed.

Results and discussion

Fungal identification and growth rates

A total of 28 isolates from seven different tree species in Korea were isolated. As shown in Table 1, nine species, including three unknown *Cladosporium* species, were identified based on genotypic characteristics. *Cladosporium cladosporioides* was the dominant species in this study. It was followed by *C. rectoides*, *C. perangustum*, *C. pseudocladosporioides*, *C. tenuissimum*, and *C. ramotenellum*. *Cladosporium cladosporioides* is a very common species that occurs on herbaceous and woody plants. This species was previously isolated from CCA-treated radiata pine boards (Kim et al. 2007), Japanese red pine logs (Kim et al. 2001), and from rice and persimmon (Kwon and Park 2003) in Korea. It had already been reported to cause plant diseases in Korea (The Korean Society of Plant Pathology 2009). In contrast, although *C. pseudocladosporioides*, *C. rectoides*, and *C. tenuissimum* were isolated from several plants in Korea (Bensch et al. 2010), all species identified in this study, except *C. cladosporioides*, have not yet been isolated from woods in Korea.

Cladosporium colonies on PDA, MEA, and OA reached an 11–69 mm diameter after 14 days. In three kinds of media, the growth rates were similar for the same strain. However, *C. perangustum* had the fastest growth on OA and *C. rectoides* had the slowest growth on the same medium. Among the strains cultured over the three types of media, *C. perangustum* had the slowest colony growth (1.6–2.2 mm day^{-1} on PDA; 0.8-2.0 mm day^{-1} on MEA; 3.0 mm day^{-1} on OA) and *C. pseudocladosporioides* had the fastest colony growth (4.4–4.6 mm day^{-1} on PDA; 4.4–4.6 mm day^{-1} on MEA; 4.3–4.7 mm day^{-1} on OA). Although colony colors varied with species and culture media, most species of *Cladosporium* were generally olive green to dull green. However, *Cladosporium* sp. 3 produced brownish spores and had extremely fast growth (7.1 mm day^{-1}) on MEA. In general, *Cladosporium* colonies are slow growing. Their colony colors are mostly greenish, but their colors are also sometimes gray, buff, or brown.

Phylogenetic analysis

Phylogenetic analyses, based on the ITS gene sequences, showed that most *Cladosporium* species were included in the *C. cladosporioides* complex. This was supported by the 100 posterior probability value (Figure 1). However, the resolution was low. To settle the limited resolution of the ITS region, a phylogenetic tree combined with the ITS, ACT, and TEF alignment was shown (Figure 2). The combined data matrix contained 41 taxa (including the one outgroup) and 1001 characters. However, *Cladosporium* sp. 1 was not included in the combined phylogeny, due to its lack of ACT sequence data. Each *Cladosporium* species was monophyletic and established its own clade, except *C. cladosporioides*. This species was separated into two distinct clades (Figure 2). These results imply that phylogenetic analysis, based on a single gene region data, is indistinguishable for *Cladosporium* identification. As a result, multilocus sequence analysis is required.

As shown in Figure 2, an unknown *Cladosporium* sp. 2 was clustered with *C. ramotenellum* in the same clade. However, the species did not match any of the *C. ramotenellum*

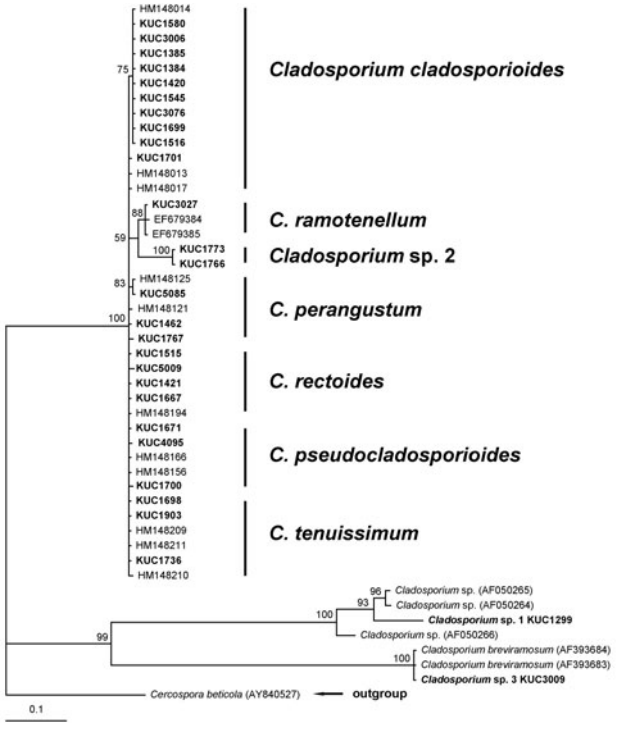

Figure 1 Consensus phylogram of 40 000 trees resulting from a Bayesian analysis of 47 sequences in ITS alignment. The tree was rooted to the sequence of *Cercospora beticola* strain CPC 11557 (GenBank accession number AY840527).

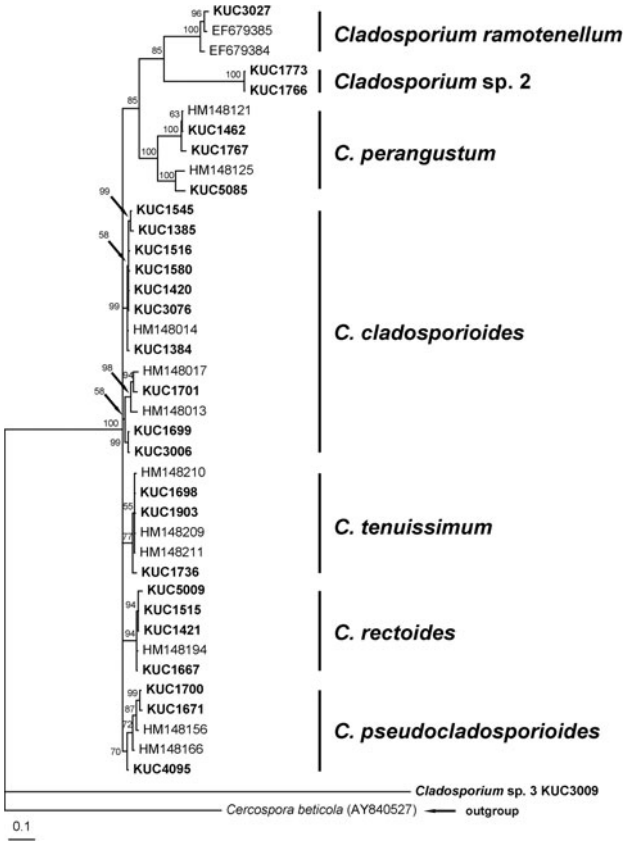

Figure 2 Consensus phylogram of 2313 trees resulting from a Bayesian analysis of 41 sequences in a combined ITS, ACT, and TEF alignment. The tree was rooted to sequences of *Cercospora beticola* strain CPC 11557 (GenBank accession numbers AY840527, AY840458, AY840494, respectively, for ITS, ACT, and TEF).

sequences in GenBank. Further studies are needed to identify this unknown species. Both unknown *Cladosporium* sp. 1 and 3 were placed away from the main *Cladosporium* clade and formed the basal part of the tree with an outgroup, *Cercospora beticola* (Figure 1). Further studies are needed to clearly identify these species.

Wood discoloration characteristics

The results of the wood discoloration tests on sapwood blocks of Japanese red pine and radiata pine are listed in Table 1. Each *Cladosporium* species showed a different level of discoloration to wood blocks and two wood species differed in the susceptibility to *Cladosporium* species, except one of the unknown species 2 isolates and unknown species 3, which showed the same discoloration rate on both blocks. Although each strain exhibited a bit of variation in discoloration rate within the same species, the blocks of Japanese red pine were generally more susceptible to *Cladosporium* than those of radiata pine. *Cladosporium* species identified to the species level had a discoloration degree of 5 within 21 days on Japanese red pine. However, except one of the unknown species 2 isolates, all unknown species showed a rate lower than 5 within the same period. A similar result was observed from the discoloration test of genus *Penicillium* (Jang et al. 2011). Because more abundant nutrients, such as sugars or nitrogenous compounds in Japanese red pine, were responsible for mold susceptibility (Jang et al. 2011), these results were considered.

Cladosporium tenuissimum and *C. cladosporioides* had the highest level of discoloration within 14 days on both blocks. Furthermore, within 7 days, they reached the highest level on radiata pine block. Bensch et al. (2010) reported that the two species are morphologically very similar and have often been misidentified and confused. In this study, however, these species were clearly distinguished by the phylogenetic analysis (Figure 2).

Most *Cladosporium* species exhibited scattered spores with olive to dark khaki colors over the entire wood surface. However, some species showed different patterns. *C. pseudocladosporioides* was floccose with gray to dark gray color. *Cladosporium* sp. 1 discolored the specimens with a zonate type. In addition, *Cladosporium* sp. 3 had a brownish color. These results were similar to the patterns observed on media.

It has been reported that *Cladosporium* species could cause discoloration in treated board (Choi 2004; Kim et al. 2007). Their discoloration ability on untreated and treated wood is an important matter. Since *Cladosporium* species are diverse and have different wood discoloration abilities, further study is required to properly prevent their growth on wood.

Conclusions

The diversity of the *Cladosporium* species inhabiting woods in Korea was investigated, based on the ability of the species to discolor sapwood blocks. By a multilocus DNA sequence analysis, the *Cladosporium* species identity was confirmed. Nine species were identified, including three unknown *Cladosporium* species. *C. cladosporioides* was dominant. All species except *C. cladosporioides* were observed in wood for the first time in Korea. In the discoloration test, both *C. tenuissimum* and *C. cladosporioides* had the strongest wood discoloration ability. Overall, the conclusion is that particular attention needs to be paid to *C. cladosporioides* on wood surfaces.

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