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Phylogenetic analysis of major molds inhabiting woods. Part 4. Genus *Alternaria*

Abstract: Twenty-four *Alternaria* strains have been isolated from wood samples in Korea and submitted to phylogenetic analyses. The gene trees generated from the ITS and histone gene region sequences revealed that, among the genus *Alternaria*, two species, *Alternaria alternata* sensu lato (s.l.) and *Alternaria tenuissima*, are involved in wood discoloration. In addition, the histone gene was useful as a marker for differentiating between *A. alternata* s.l. and *A. tenuissima*.

Keywords: *Alternaria*, histone, ITS, phylogeny, wood discoloration

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Introduction

The genus *Alternaria* Nees is a dictyosporic genus of the class Hyphomycetes belonging to the Deuteromycota (Cho et al. 2001). Dictyosporic hyphomycetes have transverse and longitudinal or oblique conidium septa (Zhao 2003). *Alternaria* conidia generally produce dark brown, green-black, or black colonies. This genus is ubiquitous and found on an extensive range of substrates worldwide. As plant pathogens, *Alternaria* species have a wide range of hosts. In addition, they are among the most common airborne allergens and occur on wood products with a wide range of uses. Seifert (1999) differentiated the sapstain fungi and arranged *Alternaria alternata* in the "dark molds," which stain the wood tissue. Consequently, *Alternaria* is associated with economic losses to wood users.

In the literature, many taxonomists have distinguished *Alternaria* species by morphologic analyses. However, the species have highly variable conidial morphologies and polymorphy in pure cultures as well as similar morphologic characteristics. The distinct differentiation of these species is somewhat confusing for nontaxonomists. Molecular identification techniques are suited to distinguish both morphologically similar and diverse species (Kang et al. 2002; Park et al. 2008). The existence of large morphologic categories within *Alternaria* has been supported by phylogenetic analyses (Andrew et al. 2009).

The aim of this study was to understand the diversity of *Alternaria* species isolated from various sites and wood species in Korea based on phylogenetic analysis.

Materials and methods

Fungal isolation and cultural characteristics

As shown in Table 1, fungi were isolated from chromate copper arsenate (CCA)-treated wood and creosote-treated wood and from specimens of Korean pine (*Pinus koraiensis*), Japanese red pine (*Pinus densiflora*), larch (*Larix kaempferi*), Douglas fir (*Pseudotsuga menziesii*), bamboo, tulip tree (*Liriodendron tulipifera*), and Norway spruce (*Picea abies*). These wood samples described in our previous publications were discolored by various molds (Huh et al. 2011; Jang et al. 2011; Lee et al. 2012, 2013). Fungal isolation and purification were carried out as described by Huh et al. (2011). Culture characteristics such as growth rates and colony pigmentation were determined as described by Lee et al. (2013).

DNA extraction, PCR, and DNA sequencing

The genomic DNA for the phylogenetic analysis was extracted according to Huh et al. (2011). The ITS region was amplified using ITS universal primer set ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′)/ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), as described by White et al. (1990) and Gardes and Bruns (1993). PCR amplification was carried out for 7 min initial denaturation, 30 cycles of 95°C for 40 s,

Table 1 *Alternaria* isolates identification based on the ITS and histone sequence data and their cultural characteristics.

Phylospecies	Isolate number[a]	GenBank accession number[b] ITS, histone	Source[c]	Sample location[d]	Growth rate[e]	Colony color on PDA, V8 agar[f]
A. alternata s.l.	KUC1572	KF051228, KF051251	LLK	YJ	5.3±0.2	Olive gray, gray
	KUC1574	KF051230, KF051253	LLK	YJ	5.3±0.1	Olive gray, gray
	KUC3072	KF051234, KF051257	CCATW	SU	8.2±0.6	Olive gray, gray
	KUC5004	KF051239, KF051263	CTW	GM	8.8±0.0	Olive gray, gray
	KUC5206	KF051246, KF051270	LLT	GJ	7.2±0.2	Olive gray, gray
A. tenuissima	KUC1369	KF051224, KF051247	LPK	YJ	6.4±0.4	Greenish gray, dark gray
	KUC1410	KF051225, KF051248	LPD	BH	6.1±0.1	Dull green, dark green
	KUC1514	KF051226, KF051249	LPK	GP	5.7±0.0	Dark gray, dark gray
	KUC1543	KF051227, KF051250	LPK	GP	7.1±1.4	Greenish green, gray
	KUC1573	KF051229, KF051252	LLK	YJ	9.9±0.0	Dark gray, pearl white
	KUC1576	KF051231, KF051254	LLK	YJ	6.9±0.2	Greenish gray, dark gray
	KUC1901	KF051232, KF051255	LPM	OY	9.6±0.6	Dark gray, dark gray
	KUC3071	KF051233, KF051256	CCATW	SU	7.5±1.1	Dark gray, dark gray
	KUC4035	HM008926, KF051258	B	JJ	6.1±0.0	Platinum, platinum
	KUC4059	KF051235, KF051259	B	JJ	7.8±0.9	Medium gray, dark gray
	KUC5001	KF051236, KF051260	CTW	GM	6.1±0.0	Greenish gray, dark gray
	KUC5002	KF051237, KF051261	CTW	GM	5.3±0.4	Dark green, greenish gray
	KUC5003	KF051238, KF051262	CTW	GM	8.5±0.6	Dark gray, dark gray
	KUC5005	KF051240, KF051264	CTW	GM	9.6±0.6	Dark gray, dark gray
	KUC5071	KF051241, KF051265	LPA	IC	9.9±0.0	Dark gray, gray
	KUC5072	KF051242, KF051266	LPA	IC	5.9±0.4	Dull green, gray
	KUC5074	KF051243, KF051267	LPA	IC	7.0±0.1	Greenish gray, dark gray
	KUC5117	KF051244, KF051268	LPA	IC	8.4±0.7	Medium gray, light gray
	KUC5143	KF051245, KF051269	LPA	IC	7.8±0.9	Dark gray, dark gray

[a]KUC, Korea University Culture Collection (Seoul, Korea).
[b]GenBank accession numbers of the ITS and histone sequences.
[c]B, bamboo; CCATW, CCA-treated wood; CTW, creosote-treated wood; LLT, log *L. tulipifera*; LLK, log *L. kaempferi*; LPA, log *P. abies*; LPD, lumber *P. densiflora*; LPK, lumber *P. koraiensis*; LPM, log *P. menziesii*.
[d]BH, Bonghwa; GJ, Gangjin; GM, Gwangmyeong; GP, Gapyeong; IC, Incheon; JJ, Jinju; OY, Onyang; SU, Seoul; YJ, Yeoju.
[e]The colony diameter (mm day^{-1}) on PDA medium at 25°C.
[f]The colony color on PDA and V8 agar medium after 14 days at 25°C.

51°C for 40 s, 72°C for 1 min, and 7 min final extension using the Accupower PCR premix kit (Bioneer, Daejeon, Korea). The additional amplification of the histone H3 gene was performed by means of primer set H3-1a (5′-ACTAAGCAGACCGCCCGCAGG-3′)/H3-1b (5′-GCGGGCGAGCTGGATGTCCTT-3′) (Glass and Donaldson 1995), and PCRs were performed according to Glass and Donaldson (1995). The amplicons were detected, purified, and sequenced as described by Huh et al. (2011). The sequences obtained in this study were deposited in GenBank under the accession numbers indicated in Table 1.

Phylogenetic analysis

The sequences generated from each sample in this study were aligned as described by Lee et al. (2012). *Alternaria dauci* (GenBank accession numbers JF417685 and JX213317 for ITS and histone, respectively) and *Alternaria solani* strains (GenBank accession numbers JF417687 and JX213319 for ITS and histone, respectively) served as outgroups. The ITS region offers limited resolution of *Alternaria* species; therefore, the results were compared with those obtained by the histone region to ensure clade stability. The ITS and histone data sets were analyzed separately, and a combined phylogenetic analysis was conducted.

Results and discussion

Fungal identification and morphologic characteristics

A total of 24 *Alternaria* isolates from seven different tree species in Korea were isolated. As shown in Table 1, only two species, *A. alternata* sensu lato (s.l.) and *Alternaria tenuissima* (Kunze) Wiltshire, were identified based on genotypic characteristics. *A. tenuissima* was the dominant species isolated from wood samples. According to the Korean Society of Plant Pathology, 42 *Alternaria* species, including the above 2 species, have been isolated from various plants. However, as mentioned above, no other

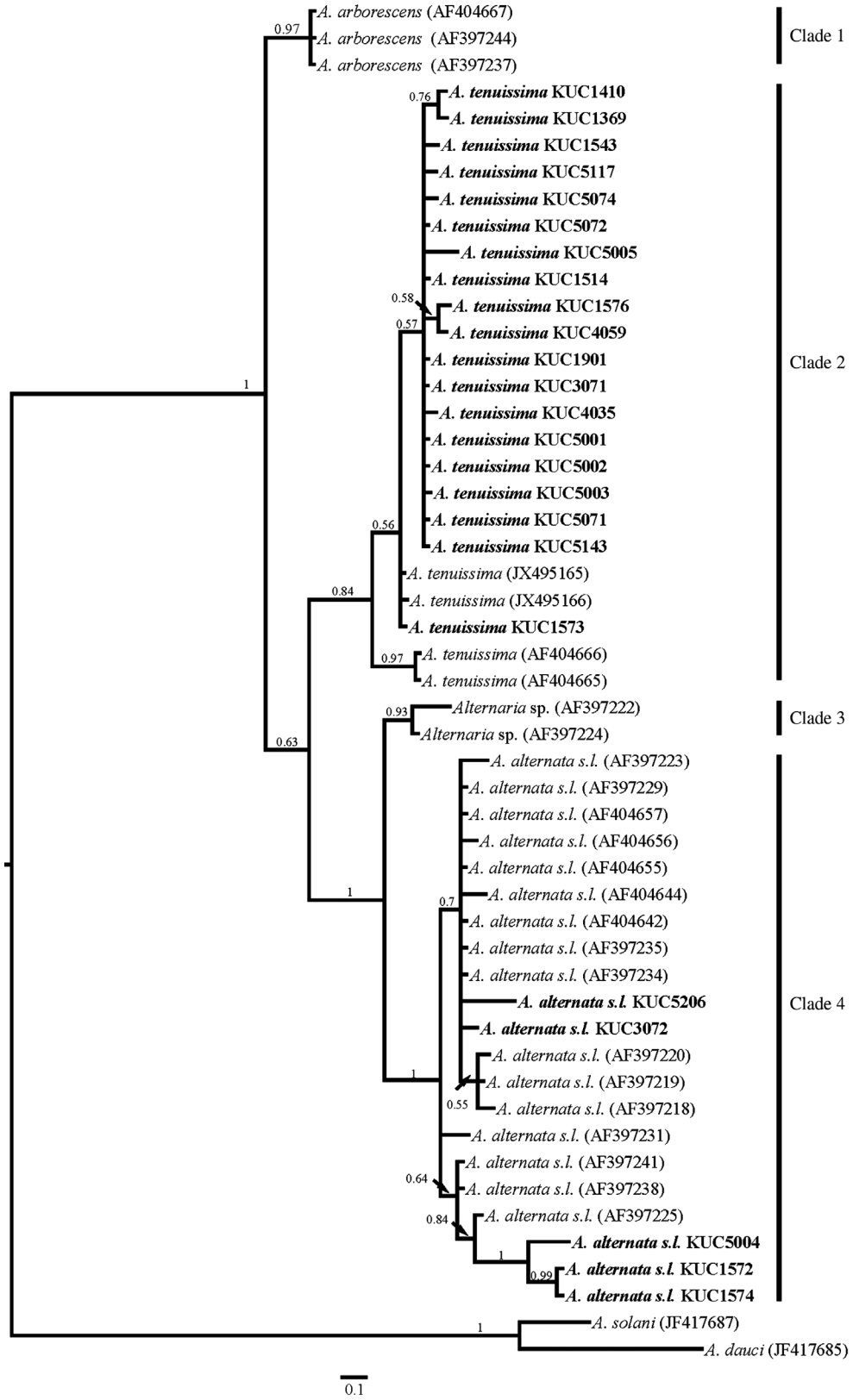

Figure 1 Consensus phylogram of 15,000 trees resulting from a Bayesian analysis of 51 sequences using the combined ITS and histone alignment.

The tree was rooted to the sequences of *A. solani* (GenBank accession numbers JF417687 and JX213319 for ITS and histone, respectively) and *A. dauci* (GenBank accession numbers JF417685 and JX213317 for ITS and histone, respectively).

Alternaria species were isolated from wood samples in this study. In addition, several researchers failed to isolate *Alternaria* species other than *A. alternata* or *A. tenuissima* from wood or wood products (Yang 2005; Vukojević and Grbić 2010; Andersen et al. 2011). Thereby, the primary source of these two species could be woods or wood products as well as these species may play an important role in their discoloration.

As shown in Table 1, the growth patterns of the two *Alternaria* species were not distinguishable. All isolates had 5.3–9.9 mm diameter of the growth rate on PDA. However, *A. alternata* s.l. produced olive gray and gray colonies on PDA and V8 agar medium, respectively, and *A. tenuissima* usually produced green to gray colonies on both PDA and V8 agar.

Phylogenetic analysis

It was possible to clearly differentiate between the two species by phylogenetic analysis. Although the ITS sequence tree showed that two species were in the same clade, the histone gene analysis was able to distinguish more taxa. The ITS sequence tree showed the large monophyletic clade assembled with a strong posterior probability value (p.p. 1.0). The clade comprised three species, *A. alternata* s.l., *Alternaria arborescens*, and *A. tenuissima*, as well as *Alternaria* sp. Based on the histone gene analysis, the isolates were separated into three groups. The histone gene tree, however, had limited resolution. The tree was similar to the combined gene tree constructed based on the alignment of ITS and histone data sets (Figure 1), except for the resolution of the relationships among clades 1, 2, and 3–4. The combined data matrix contained 51 taxa (including the outgroups) and 1193 characters. The 50% majority rule consensus tree generated by Bayesian analysis revealed two major clades comprising four smaller clades. The clades of the *A. arborescens* (clade 1), *A. tenuissima* (clade 2), *Alternaria* sp. (clade 3), and *A. alternata* s.l. (clade 4) received high posterior probability values. The major clade comprising clade 1 received a high p.p. (0.97). The other major clade comprised clades 2–4. Clade 2 consisted of a large number of *A. tenuissima* isolates from various wood species and received a high p.p. (0.84). Clades 3 and 4 comprised *Alternaria* sp. and *A. alternata* s.l., respectively. Consequently, most isolates were identified as *A. tenuissima*, and only five isolates were assigned to *A. alternata* s.l. Additionally, no correlation was found between *Alternaria* species identification and host or geographic origin in the studied samples. Roberts et al. (2000) concluded that the ITS region of the rDNA gene

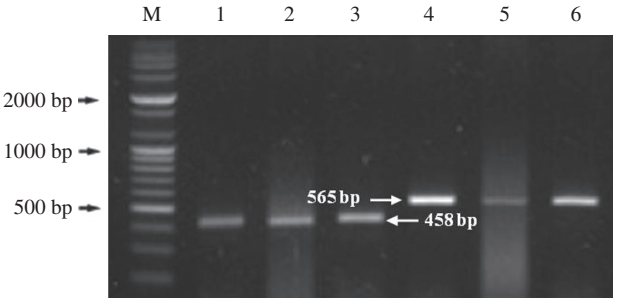

Figure 2 Agarose gel PCR of the partial histone gene.
Lanes: M1, 100-bp DNA marker: 1, *A. alternata* s.l. KUC1572; 2, *A. alternata* s.l. KUC1574; 3, *A. alternata* s.l. KUC5004; 4, *A. tenuissima* KUC1543; 5, *A. tenuissima* KUC4035; 6, *A. tenuissima* KUC4059.

was not appropriate for use to assess variability among small-spored *Alternaria* species and suggested that more informative genes would be needed for sequence analysis. Oddly, the resolution power of other regions was also poor, such as the 28S ribosomal DNA, translation elongation factor, *Alternaria* allergen α1, mitochondrial small subunit, endopolygalacturonase, and glyceraldehyde-3-phosphate dehydrogenase genes (data not shown). Interestingly, as shown in Figure 2, PCR amplicons of the histone gene region from *A. alternata* s.l. and *A. tenuissima* showed bands of different sizes, 458 and 565 bp, respectively. *A. tenuissima* KUC4035 (Figure 2, lane 5) was originally identified as *A. alternata* (Kim et al. 2011). This isolate, however, was reidentified in our phylogenetic analysis and correspondingly renamed.

This particular feature indicates that the histone gene can differentiate both species; consequently, this study suggests that the histone gene could be an ideal marker for differentiating *A. alternata* s.l. and *A. tenuissima*. Nevertheless, as explained in Kang et al. (2002), the histone data sets could not be used to distinguish additional taxa in *A. alternata* s.l., and it was unable to relate the morphologic and cultural differences to the species. They furthermore explained that the clade of *A. alternata* s.l. seemed to contain several different species. Therefore, further studies should be pursued based on other molecular or chemotaxonomic approaches.

Conclusions

In this study, a total of 24 *Alternaria* isolates from various sites and wood species were identified. Phylogenetic analysis of the isolates was performed with the ITS and histone gene region sequences. The gene trees generated from the individual and combined data sets based on the Bayesian

analysis have been interpreted that two *Alternaria* species, *A. alternata* s.l. and *A. tenuissima*, are involved in the discoloration of wood in Korea. According to this study, the histone gene could be a useful marker for differentiating *A. alternata* s.l. and *A. tenuissima*.

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References

Andersen, B., Frisvad, J.C., Søndergaard, I., Rasmussen, I.S., Larsen, L.S. (2011) Associations between fungal species and water-damaged building materials. Appl. Environ. Microbiol. 77:4180–4188.

Andrew, M., Peever, T.L., Pryor, B.M. (2009) An expanded multilocus phylogeny does not resolve morphological species within the small-spored *Alternaria* species complex. Mycologia 101: 95–109.

Cho, H.S., Kim, B.R, Yu, S.H. (2001) Taxonomic studies on *Alternaria* in Korea (1). Mycobiology 29:27–42.

Gardes, M., Bruns, T.D. (1993) ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Mol. Ecol. 2:113–118.

Glass, N.L., Donaldson, G.C. (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. 61:1323–1330.

Huh, N., Jang, Y., Lee, J., Kim, G.-H., Kim, J.-J. (2011) Phylogenetic analysis of major molds inhabiting woods and their discoloration characteristics. Part 1. Genus *Trichoderma*. Holzforschung 65:257–263.

Jang, Y., Huh, N., Lee, J., Lee, J.S., Kim, G.-H., Kim, J.-J. (2011) Phylogenetic analysis of major molds inhabiting woods and their discoloration characteristics. Part 2. Genus *Penicillium*. Holzforschung 65:265–270.

Kang, J.-C., Crous, P.W., Mchau, G.R.A., Serdani, M., Song, S.-M. (2002) Phylogenetic analysis of *Alternaria* spp. associated with apple core rot and citrus black rot in South Africa. Mycol. Res. 106:1151–1162.

Kim, J.-J., Lee, S.-S., Ra, J.-B., Lee, H., Huh, N., Kim, G.-H. (2011) Fungi associated with bamboo and their decay capabilities. Holzforschung 65:271–275.

Lee, Y.M., Jang, Y., Kim, G.-H., Kim, J.-J. (2012) Phylogenetic analysis and discoloration characteristics of major molds inhabiting woods. Part 3. Genus *Cladosporium*. Holzforschung 66:537–541.

Lee, Y.M., Hong, J.-H., Lee, H., Ahn, B.J., Kim, G.-H., Kim, J.-J. (2013) Phylogenetic analysis of the genus *Fusarium* and their antifungal activity against wood-decay and sapstain fungi. Holzforschung 67:473–478.

Park, M.S., Romanoski, C.E., Pryor, B.M. (2008) A re-examination of the phylogenetic relationship between the causal agents of carrot black rot, *Alternaria radicina* and *A. carotiincultae*. Mycologia 100:511–527.

Roberts, R.G., Reymond, S.T., Andersen, B. (2000) RAPD fragment pattern analysis and morphological segregation of small-spored *Alternaria* species and species group. Mycol. Res. 104:151–160.

Seifert, K.A. (1999) Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. *Ceratocystis* and *Ophiostoma*. In: Taxonomy, Ecology, and Pathogenicity, 2nd. ed. Eds. Wingfield, M.J., Seifert, K.A., Webber, J.F. Am. Phytopath. Soc. Press, Minnesota. pp. 141–151.

Vukojević, J., Grbić, M.L. (2010) Moulds on paintings in Serbian fine art museums. Afr. J. Microbiol. Res. 4:1453–1456.

White, T.J., Bruns, T., Lee, S., Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR protocols: a guide to methods and applications. Eds. Innis, M.A., Gelfand, D.H., Shinsky, J.J., White, T.J. Academic Press, San Diego, CA. pp. 315–322.

Yang, D.-Q. (2005) Isolation of wood-inhabiting fungi from Canadian hardwood logs. Can. J. Microbiol. 51:1–6.

Zhao, G.-Z. (2003) Taxonomic studies on 20 dictyosporic Hyphomycete genera from China and molecular systematics of represented species of 5 allied genera. Ph.D. thesis, Shandong Agricultural University.