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Characterization of a Sapstaining Fungus, *Ophiostoma floccosum*, Isolated from the Sapwood of *Pinus thunbergii* in Korea

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An *Ophiostoma* fungus was isolated from a stump of *Pinus thunbergii* in a forest on the West coast of Korea. Microscopic analysis using a light microscope, a stereo microscope, and a scanning electron microscope revealed that it had morphological features of *Pesotum* and *Sporothix* synanarmorphs. Based on the β-tubulin gene sequence analysis, the fungus was identified as the anamorph of *Ophiostoma floccosum*. Mycological properties of the species including its growth properties on different culture media were described.

KEYWORDS : β-tubulin gene, *Ophiostoma floccosum*, *Pinus thunbergii*

The ascomycete genus *Ophiostoma* includes many economically important species causing tree diseases and discoloration of wood. Taxonomically, the *Ophiostoma* genus is mainly characterized by dark, globose perithecia with a neck of a variable length, rapidly evanescent asci, and small and unicellular ascospores, usually with a mucilaginous sheath. Within the genus, one of the difficult groups to identify is the *Ophiostoma piceae* complex. More than ten *Ophiostoma* species have been classified in this complex group. Anamorphic stages of the species in the *O. piceae* complex resemble the anamorphs of *Pesotum*, *Hyalorhinocladiella* and *Sporothrix* with various morphological differences (Seifert *et al*., 1993; Wingfield *et al*., 1993; Harrington *et al*., 2001). Because they share similar morphological features and each species can have one or more of several anamorphs, it is not easy to differentiate the *O. piceae* complex species based only on morphological characters. To assist in the differentiation of the *O. piceae* complex species, molecular methods including PCR and nucleotide sequence analysis of the ribosomal RNA gene and/or the β-tubulin genes have been used (Kim *et al*., 1999; Uzunovic *et al*., 2000; Harington *et al*., 2001; Chung *et al*., 2006).

Wood discoloration occurring in the sapwood of logs is called sapstain or bluestain. Some species in the *O. piceae* complex are known as sapstain species that are responsible for bluish-black or black discoloration of wood. Since they have melanin pigment in their mycelia, their presence in logs and lumber decreases the value of wood products, although they do not reduce the strength of the wood (Schirp *et al*., 2003). Thus, the forest product industry spends millions of dollars annually to control sapstaining fungi (Abraham *et al*., 1993).

During the survey of fungi in 2005 from the stumps of pine trees that were cut in a forest located in Taean, Chungchungnamdo, Korea, sapstained stumps were found from Japanese black pine and fungi were isolated from the stained stumps. One of the isolated fungi showed morphological features similar to *Pesotum* and *Sporothrix* synanarmorphs that are known to be observed in the *Ophiostoma piceae* complex. In this study we identified the fungal species as the anamorph of *Ophiostoma floccosum*, a sapstaining species that is found in conifer trees worldwide. The morphological and colony characters of the anamorph of the identified *Ophistoma* species were described including information on its β-tubulin gene sequences.

Materials and Methods

Fungal isolation and culture conditions. In 2005, wood discs were cut from a discolored stump of *Pinus thunbergii* (Fig. 1) grown in a forest in Taean, Chungnam, Korea. After washing with tap water, the sapwood of the discs was chipped into small pieces. The wood pieces were surface-sterilized with 0.5% chlorine lax solution for 2 min, washed twice with sterile water, and air dried. The dried wood pieces were placed on MEA (2% malt extract agar, BD Science, USA) supplemented with cycloheximide (200 μg/*ml*) and incubated for 10 days at room temperature. Fungal isolates were obtained by taking mycelial tips grown out from the incubated wood pieces. Single spore isolates were prepared from the obtained isolates grown on 2% MEA at 25°C for seven days. All the isolated fungi were stored at −80°C in 10% glycerol for long-term storage and in water at 4°C for short-term storage.

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Fig. 1. The stained wood stump of Japanese black pine used for DKM0514 isolation.

Observation of cultural and morphological characteristics. To identify optimal growth media, pre-cultured isolates were transferred to the center of each of PDA, MEA, OA (oatmeal agar, BD Science, USA) and OMEA (2% Oxoid malt extract agar, Oxoid, UK) media, respectively, and grown for 9 days at 25°C. Mycelial growth was recorded by measuring the diameters of the colonies. Light microscopic images of the morphological features of the isolates were examined under a phase-contrast microscope (Karl Zeiss, Axioskop 40) after growing the culture on 2% MEA at 25°C for 5~7 days. For scanning electron microscopy (SEM), the fungal isolate was grown for 5~7 days at 25°C on 2% MEA plates overlaid with cellophane (Bio-Rad Laboratories, Canada). Pieces of cellophane were fixed in 2% glutaraldehyde buffered in 0.1 M cacodylate buffer for 16 h and 1% osmium tetroxide in 0.1 M phosphate for 1 h. Samples were subsequently washed in 0.05M cacodylate buffer, dehydrated in a series of ethanol washes (50% for 20 min, 75% for 20 min, 90% for 20 min, 95% for 20 min and 100% for 20 min), passed through ethanol-isoamylacetate, dried with a Hitachi critical point dryer and coated with platinum-palladium at 25 nm with an Hitachi E-1030 ion sputter. The specimens were examined with a Hitachi S-4300 scanning electron microscope operating at 15 kV.

Nucleic acid preparation, PCR amplification and nucleotide sequencing. For fungal genomic DNA, fungi were grown for 5~7 days on 2% MEA plates overlaid with cellophane (Bio-Rad Laboratories, USA). Fungal genomic DNA for PCR was obtained from the mycelium using the drilling method described by Kim *et al.* (1999). The β-tubulin gene was amplified using the primers T10 and BT12 (O'Donnel and Cigelnik, 1997; Kim *et al.*, 2003). Amplification was done in a Gene Amp-950 thermal cycler (ABI, USA). Sequencing was performed on an ABI 3700 automated sequencer (Perkin-Elmer Inc., USA). The obtained nucleotide sequences were searched through BLASTN at GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/).

Molecular phylogenetic analysis. Sequences were manually edited using the Chromas v2.31 program and aligned using the ClustalW 2 program. Reference sequences of related taxa were obtained from the GenBank database. The aligned sequences were analyzed with PAUP 4.0 beta 10 (Swofford, 2003). The phylogenetic trees based on β-tubulin gene sequences were constructed by the neighbor-joining method. Bootstrap values were generated with 1000 replicate heuristic searches.

Results and Discussion

Initially. several fungal isolates were obtained from the sapwood pieces of a stained stump of Japanease black pine. They all grew well in the cycloheximide-supplemented MEA media, indicating that they are cycleheximide tolerant. They showed the same morphology and growth pattern, thus, one of the fungal isolates coded as DKM 0514 was used for identification in this work.

The isolate formed pale or red synnemata and droplets of conidia at the apex the synnemata (Fig. 2A~2F). Conidia masses on synnemata were yellow (Table 1). The isolate also formed mononema (Fig. 2B). The shape and size of conidia on synnema and mononema are given in Table 1. These features of synnemata are similar to synnema-forming species of the *Ophiostoma piceae* complex. Recently, Chung *et al.* (2006) compared morphological characters of seven species of the *O. piceae* complex, including *O. breviusculum*, *O. canum*, *O. floccosum*, *O. piceae*, *O. quercus*, *O. setosum*, and *O. subalpinum*. Among these compared species reported from conifers, the characters of *O. floccosum* were most matched with those of DKM 0514. Especially, the red-brown synnema and yellow color of the conidia mass found in DKM 0514 were considered to be distinct features of *O. floccosum* by Harrington *et al.* (2001).

O. floccosum (Mathiesen) Hunt (anamorph: *Pesotum aureum* (Hedgecock) McNew et Harrington), one of the common sapwood-staining species (Hunt, 1956; Harrington *et al.* 2001), is known to have a *Sporothrix* type anamorph. Thus, we examined DKM 0514 to find it has a *Sporothrix* type anamorph. A *Sporothrix* synanamorph with denticles and secondary conidia was observed from DKM 0514 by scanning electron microscopy (Fig. 2G~2I). *O. floccosum* is a heterothallic species, as it can produce perithecia only through cross of two different mating type partners. In this study DKM 0514 itself did not form a perithecium, suggesting it is a heterothallic species. Consequently, we could not observe a telemorph of DKM 0514 (Table 1). Thus, DKM 0514 was considered as an

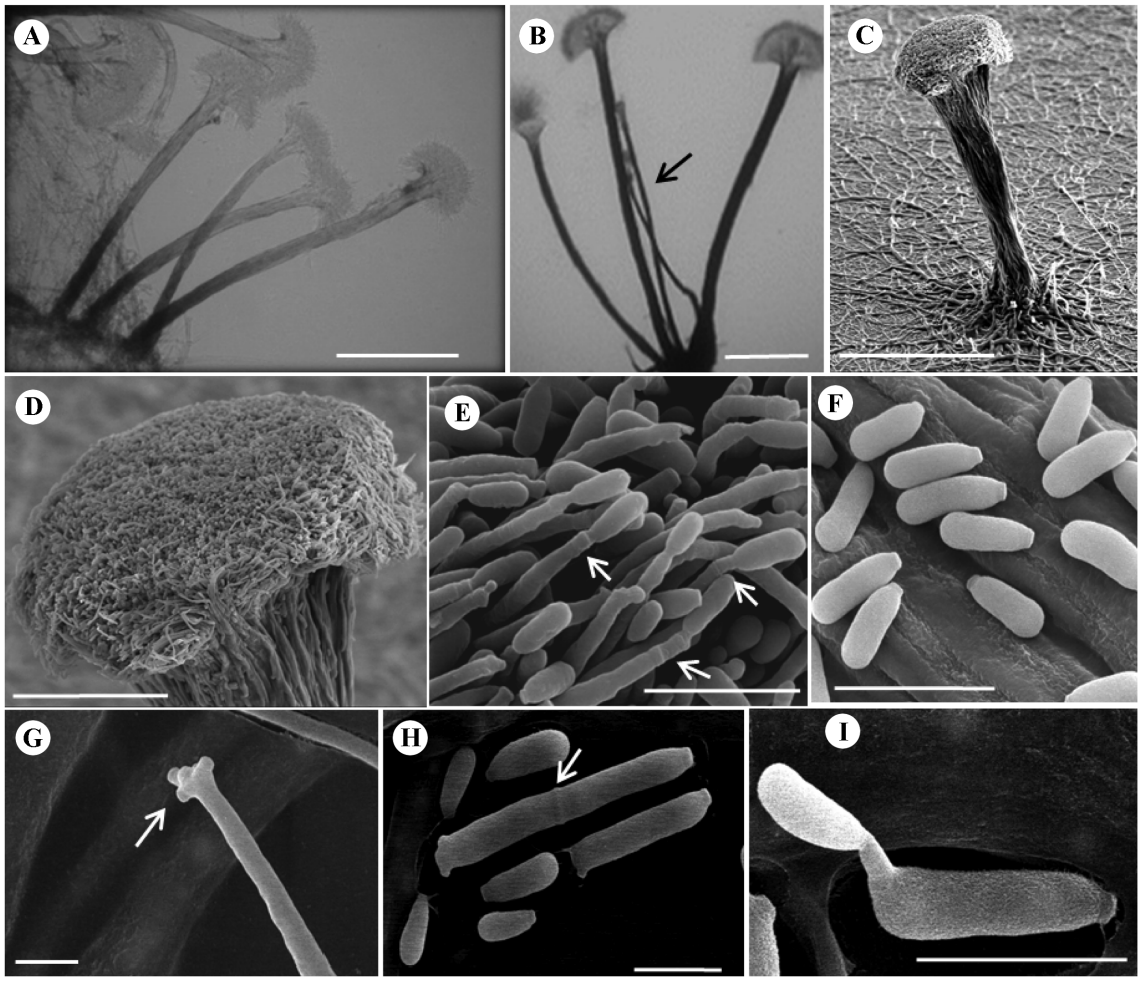

Fig. 2. Morphological features of the fungal isolate DKM0514. A and B, light micrograph of synnemata and mononema (arrow); C and D, scanning electron micrograph of a synnema; E and F, scanning electron micrograph of conidiogenenous cell showing annellations (arrow) and conidia; G, scanning electron micrograph of denticles on apex of a conidiogenenous cell of *Sporothrix* synanamorph; H, scanning electron micrograph of aseptate and septate (arrow) conidia; I, scanning electron micrograph of a conidium with a secondary conidium. Scale bars: A = 100 μm; B = 50 μm; C = 200 μm; D = 50 μm; E, F, H, I = 5 μm; G = 3 μm.

Table 1. Comparison of morphological characters of the isolate DKM0514 to those of *Ophiostoma piceae* and *O. floccosum*

Characters	*O. piceae*[a]	*O. floccosum*[a]	DKM0514
Perithecia			
Width of base (μm)	100~180 (220)	155	
Length of neck (μm)	600~1600	940~1600	NF
Ascospores			
Shape	reniforma	kidney-shaped	
Size (μm)	(3.5~) 2.5 × 1.5	3.5 × 1.7	NF
Synnemata			
Color	dark brown	pale brown, red brown	pale brown, red brown
Length (μm)	200~700 (800)	up to 300~500	300~400
Conidia on synnema			
Shape	oblong	cylindrical to ellipsoid	cylindrical to ellipsoid
Size (μm	(4.5~) 2.5 × 1 (~1.5)	3~5 × 1.5~2	3~4 × 1~2
Conidia on monomema			
Shape	clavate	ovoid, some elongate	ovoid
Size (μm)	7~28	5~8 × 2~4	4~8 × 1.5~3

[a]Data from Hunt (1956) and Harrington *et al.* (2001). NF: No formation.

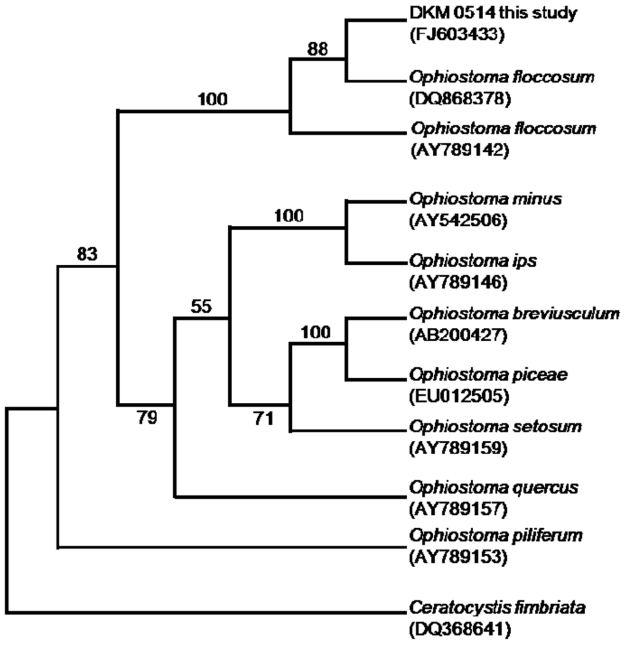

Fig. 3. Phylogenetic relationships of the fungal isolate DKM 0514 to other *Ophiostoma* species. Cladogram based on the anaysis of partial nucleotide sequence of the β-tubulin gene was generated by the neighbor-joining analysis. Numbers at nodes represent percentage of bootstrap resampling based on 1000 replicates. *Ceratocystis fimbriata* is used as an outgroup.

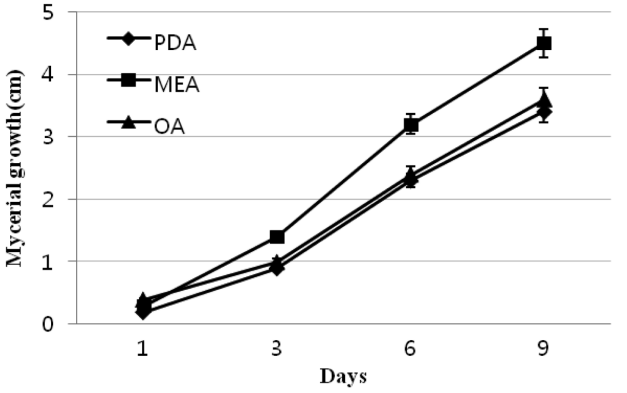

Fig. 4. Mycerial growth of the fungal isolate DKM 0514 on different media at 25°C. PDA: potato dextrose agar. MEA: malt extract agar. OA: oatmeal agar.

anamorphic isolate.

The differentiation of the *O. piceae* complex is not easy based only on morphological characters. Therefore, to confirm whether the isolate is the anamorph of *O. floccosum*, its β-tubulin gene sequence was analyzed. An 839 bp-sized nucleotide sequence was determined from the isolate DKM 0514. When the determined β-tubulin gene sequence was searched for homologous sequences in the GenBank DNA database, it matched well with the known

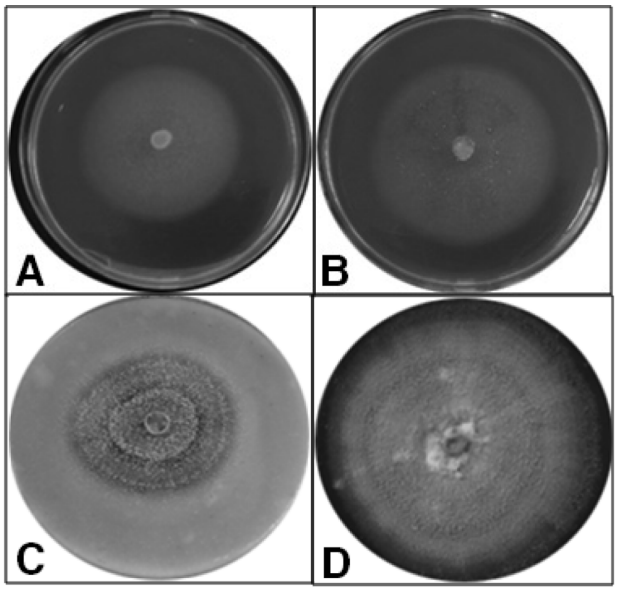

Fig. 5. Colony morphology of the isolate DKM 0514 grown at 25°C on PDA (A), MEA (B), OA (C), and OMEA (D).

β-tubulin gene sequence of *Ophiostoma floccosum* KUC 2412 (Accession number DQ868378) (100% similarity). The β-tubulin gene sequence of the isolate DKM 0514 was deposited in the GenBank under accession no. FJ603433. The phylogenetic analysis based on the β-tubulin gene sequences also revealed that DKM 0514 was placed in a clade with *O. floccosum* (Fig. 3).

The isolate DKM 0514 from this study showed better mycelial growth on MEA than on PDA and OA (Fig. 4). The colony color of *O. floccosum* DKM 0514 incubated at 25°C was creamy on PDA, creamy grayish on OA, and first white then gradually turning brownish on MEA and OMEA (Fig. 5). It produced no culture aroma and no mycelium with concentric rings that were observed as distinguishing characteristics of *O. floccosum* by Harrington *et al.* (2001).

Overall, based on the morphological characters (Table 1, Fig. 2), results of the nucleotide homology search, phylogenetic analysis (Fig. 3) of the â-tublin gene, and physiological properties, the isolate DKM 0514 was identified as the anamorph of *Ophiostoma floccosum.* This is the first report in Korea on the detailed mycological properties of the anamorph of *O. floccosum* isolated from *Pinus thunbergii*.

Acknowledgements

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