Phylogeny and Taxonomy of the *Ophiostoma piceae* Complex Associated with Bark Beetles Infesting Japanese Larch in Japan

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Contents

Chapter 1. Introduction

Japanese larch, *Larix kaempferi*, has limited native range in the central mountains of Honshu of Japan. Moreover, it was introduced to Hokkaido in the early 1900s and now 80 % of Japan's larch production occurs in these plantations (Nagamitsu et al. 2014a). Japanese larch is well known for its rapid juvenile (4-8 y) height growth (Isebrands and Hunt 1975), and its wood have been used in the pulp and paper industry (Perry and Cook 1965).

Sap staining of lumbers and pulp chips caused by blue-stain fungi might cause economic problems (Whitney 1982; Seifert 1993; Butin 1996). The stained wood has a lower market value (Held et al. 2003). One of the famous sap staining fungi is *Ophiostoma piceae* (Münch) H. & P. Sydow. *Ophiostoma piceae* was associated with bark beetles, such as *Ips subelongatus*, invading Japanese larch, and known to have weak pathogenicity against Japanese larch (Yamaguchi et al. 1991; Peng et al. 1996; Yamaoka et al. 1998).

Many ophiostomatoid fungi causing blue stain in the sapwood of conifers that dying or recently killed (Gibbs 1993; Seifert 1993). Sap or blue stain is a grey, black or bluish discoloration of sapwood caused by the presence of pigmented fungal hyphae in the tracheid (Seifert 1993). The stored logs, timber and other wood products of conifer were de-valued by blue stain (Gibbs 1993; Seifert 1993; Butin 1996).

It was well known that bark beetles (Coleoptera: Scolytidae) are commonly associated with many sapstain fungi, especially ophiostomatoid species. Most bark beetles are secondary pests that invade stressed trees, but some are primary forest pests (Wood and Bright 1992) that can kill healthy living trees (Paine et al. 1997).

There is an intimate and relatively specific association between *Ophiostoma* and their asexual morphs and bark beetles (Mathiesen-Kaarik 1953; Whitney 1982; Paine et al. 1997; Jacobs and Wingfield 2001). The sticky ascospores and conidia of blue stain fungi were adhering to the insect's exoskeleton or digested and were disseminated (Mathiesen- Käärik 1953; Francke-Grosmann 1967; Whitney 1982; Furniss et al. 1990; Paine et al. 1997). Alternatively, the host tree's defense mechanisms overcome by bark beetles with help of the associated blue-stain fungi, and then tree was killed (Lieutier 2002; Kirisits 2004).

1-1. Taxonomic history of the *Ophiostoma piceae* complex

Among the ophiostomatoid fungi, *Ophiostoma piceae* is one of the popular blue stain fungi of conifers (Brasier and Kirk 1993; Seifert 1993). *Ophiostoma piceae* was described as a coniferous sap-staining fungus (Münch 1907). Later, *O. quercus* (Georgévitch) Nannf. (reported as *O. querci*) was described as a new species from oak (Georgévitch 1926). Hunt (1956) treated *O. quercus* as a synonym of *O. piceae* based on morphological similarities between the two species. This taxonomic placement was accepted by other researchers, e.g., Griffin (1968), Olchowecki and Reid (1974), and Upadhyay (1981). However, these two species were recognized as distinct, reproductively isolated sibling species based on mating experiments (Brasier and Kirk 1993). Furthermore, other researchers suggested that *O. piceae* and *O. quercus* have different biological characters, e.g., growth ability at 32 °C on 2 % Oxoid malt extract agar (MEA) (Brasier and Stephens 1993; Przybyl and Morelet 1993; Wulf and Kowalski 1994). Moreover, *O. piceae* is differentiated from *O. quercus* on the basis of a broader synnema head and a longer and wider stipe than that of *O. quercus* (Morelet 1992, Przybyl and Morelet 1993; Halmschlager et al. 1994). Based on the DNA

analyses of randomly amplified polymorphic DNA (RAPD) and the internal transcribed spacer of the nuclear ribosomal RNA gene (ITS nrDNA) region, the two species were also recognized distinct from each other (Halmschlager et al. 1994; Pipe et al. 1995; Kim et al. 1999).

Later, morphological study and DNA analysis of the ITS nrDNA region suggested that *O. piceae*, *O. quercus*, and Dutch Elm Disease pathogens, *O. ulmi, O. novo-ulmi* and *O. himal-ulmi* are a complex of closely related *Ophiostoma* species with pigmented, synnematous asexual morph (Brasier and Kirk 1993; Brasier and Mehrotra 1995). Subsequently, another four species, *O. canum* (Münch) H. & P. Sydow, *O. floccosum* Mathiesen, *O. setosum* Uzunovic et al., and *O. catonianum* (Goid.) Goid were suggested that closely related with *O. piceae*, *O. quercus*, *O. ulmi*, *O. novo-ulmi* and *O. himal-ulmi* in the study of Harrington et al. (2001). These above-mentioned nine species were composed the *O. piceae* complex (Harrington et al. 2001) based on comprehensive study of morphological characteristics of the synnemata (i.e., color of stipe, knobs on stipe, copulated apex, color and shape of conidia), culture characteristics (i.e., aroma, growth assay at 32 °C, protoperithecia color, and concentric rings of aerial mycelium, tolerant of cycloheximide), mating compatibility tests, and phylogenetic analysis of the ITS nrDNA region.

The species in the *O. piceae* complex defined by Harrington et al. (2001) were characterized by black perithecia with slender necks and ostiolar hyphae, orange section-shaped or reniform ascospores, pesotum-like and sporothrix-like asexual morphs. Furthermore, phylogeny inferred from the ITS nrDNA region suggested two major clades, conifer clade (four conifer-inhabiting species) and hardwood clade (five hardwood-inhabiting species), in the *O. piceae* complex. The conifer-inhabited clade that with moderately supported value (75) included *O. piceae*, *O. canum*, *O.*

floccosum and *O. setosum*, while the other hardwood-inhabited clade that well-supported included *O. quercus*, *O. catonianum*, *O. ulmi*, *O. novo-ulmi* and *O. himal-ulmi*.

Based on synnematous characteristics (Ohtaka et al. 2002a; Chung et al. 2006), inter-species mating experiment and DNA analyses on the ITS nrDNA region and the partial β-tubulin gene (Chung et al. 2006), another two species, *O. subalpinum* Ohtaka & Masuya (Ohtaka et al. 2002a) and *O. breviusculum* Chung et al. (Chung et al. 2006) were reported from Japan as members of the *O. piceae* complex. Recently, a new species, *O. rachisporum* Linnakoski et al., from conifers in Finland and Russia, was added to the *O. piceae* complex, together with three more species that seemed to be members of this complex, *O. brunneum*, *O. flexuosum* and *O. distortum*, but without the pesotum-like asexual morph based on morphological study and phylogenetic analyses on the ITS nrDNA region and the partial β-tubulin gene (Linnakoski et al. 2009, 2010).

Based on comprehensive phylogenetic analysis of ITS nrDNA region and the nuclear large subunit ribosomal RNA gene region, a strongly supported group comprising 15 hardwood-inhabiting species was established and named as the *O. ulmi* complex (De Beer and Wingfield 2013). These fifteen species are *O. quercus*, *O. catonnianum*, *O. ulmi*, *O. novo-ulmi*, *O. himal-ulmi*, *O. tasmaniense*, *O. borealis*, *O. australiae*, *O. denticiliatum*, *O. undulatum*, *O. tsotsi*, *O. bacillisporum*, *O. karelicum*, *O. triangulosporum* and *O. tetropii* (Harrington et al. 2001; Grobbelaar et al. 2009a, 2010; Kamgan Nkuekam et al. 2011). This group of species was all isolated from hardwoods, producing Type A-shaped ascospore (i.e., orange-section, clavate to ovate, and lunate), and pesotum-like and sporothrix-like asexual morphs (De Beer and Wingfield 2013).

On the other hand, four conifer-inhabiting species, *O. piceae*, *O. canum*, *O. setosum* and *O. floccosum*, which were previously included in the *O. piceae* complex sensu Harrington et al. (2001), and another six species, *O. breviusulum*, *O. rachisporum*, *O. subalpinum*, *O. brunneum*, *O. flexuosum* and *O. distortum*, which were included in conifer clade of the *O. piceae* complex (Linnakoski et al. 2009, 2010), were not revealed as monophyletic in the analyses of De Beer and Wingfield (2013). Thus, they were treated as part of *Ophiostoma* sensu stricto (De Beer and Wingfield 2013). These species in the part of *Ophiostoma s. str.* has pesotum-like and/or sporothrix-like asexual morphs, and Type A-shaped ascospore.

Yin et al. (2016) newly defined *O. piceae* complex, indicating a more specific monophyletic lineage based on molecular phylogenetic analyses of ITS nrDNA region, β-tubulin, calmodulin, and translation elongation factor-1α genes and morphological studies. These species included three new species without sexual morphs: *O. nitidum*, *O. micans*, and *O. qinghaiense*, and the six species *O. piceae*, *O. breviusculum*, *O. canum*, *O. flexuosum*, *O. rachisporum*, and *O. brunneum*. The three new species are without sexual morph from Qinghai spruce (*Picea crassifoila*). They suggested that the species with known sexual morphs are all characterized by unsheathed, allantoid ascospores, and most species produce pesotum-like and sporothrix-like asexual morphs. As such, in this study, this newly defined *O. piceae* complex is applied.

1-2. Taxonomic status of the *Ophiostoma piceae* complex in Japan

In Japan, both *O. piceae* and *O. quercus* were present, but were not distinguished (Nisikado and Yamauti 1935; Ito 1973; Ohtani 1988) until De Beer et al. (2003) did

so using mating compatibility tests and ITS nrDNA region phylogeny. *Ophiostoma canum* (Masuya et al. 1999) and *O. breviusculum* (Chung et al. 2006) were also reported in Japan. *Ophiostoma piceae* is associated with bark beetles, such as *Ips subelongatus* invading Japanese larch, and is known to cause blue staining of sapwood and to have weak pathogenicity against Japanese larch (Yamaguchi et al. 1991; Peng et al. 1996; Yamaoka et al. 1998). *Ophiostoma breviusculum* is also known to be associated with *I. subleongatus* and other bark beetles invading Japanese larch (Chung et al. 2006; Yamaoka et al. 2009).

During surveys on the *Ophiostoma* species associated with bark beetles infesting Japanese larch since 1989, more isolates seemed to be species of the *O. piceae* complex were obtained from several localities at Central Honshu, Japan. For example, Yamaoka et al. (2009) reported the presence of two distinct mating populations in *O. breviusculum,* which were morphologically and ecologically indistinguishable. They reported as *O.* cf. *breviusculum*. One of the populations which was unable to mate with isolates related type specimen (YCC-519 and YCC-522) of *O. breviusculum,* is named as Group A in the present study. Group A was isolated from bark beetle and beetle galleries of *Ips subelongatus* invading Japanese larch at Nikko, Tochigi Pref., in 2001. Another group related with *O. breviusculum*, named Group B in the present study, was obtained during the survey conducted at Sugadaira, Nagano Prefecture in 2006 and reported as *O.* cf. *breviusculum* (Tokumasu 2009). It was consistently isolated from bark beetle and beetle galleries, *Dryocoetes pini*. Sexual morph of Group B was morphologically similar to *O. breviusculum* and Group A. However, colony appearance of Group B was dark-brown with a few production of synnemata on the medium. It was uncertain whether these populations were the same species or not.

The third group of isolates named Group C in this study, were consistently isolated from bark beetle and beetle galleries, *Polygraphus kisoensis*, during a survey conducted at Sugadaira, Nagano Prefecture in 2006 and reported as *O.* cf. *piceae* (Tokumasu 2009). In our preliminary study, Group C was morphologically similar to the *O. piceae* complex, but the taxonomic status of this group was uncertain.

Ophiostoma piceae is one of the most common ophiostomatoid fungi. It was reported on various conifers and globally distributed (Dowding 1969; Solheim and Krokene 1998; Uzunovic and Webber 1998). In Japan, *O. piceae* was reported to be associated with many kinds of bark beetles infesting conifers including Japanese larch (Aoshima 1965; Yamaoka et al. 1998, 2009), Yezo spruce (Tochinai and Sakamoto 1934; Aoshima 1965; Yamaoka et al. 1997), firs (Aoshima 1965), Japanese red pine (Nishikado and Yamauti 1935; Aoshima 1965), beech *(Fagus crenata* Blume) (Aoshima and Hayashi 1956; Aoshima 1965), and various other conifers and hardwoods (Ohtani 1988). They were treated as conspecific. However, for example, Yamaoka et al. (1998) mentioned that the population of *O. piceae* associated with *Ips subelongatus* (as *I. cembrae*) might have wider perithecial base. Even the Japanese population of *O. piceae* might contain cryptic species.

Since the populations mentioned above shared very similar morphological features, it was difficult to determine taxonomic status based on morphological characteristics only. Molecular phylogenic analyses using ITS nrDNA region, partial β-tubulin and EF-1α genes (Jacobs and Kirisits 2003; Chung et al. 2006; Linnakoski et al. 2008, 2009, 2010; Kamgan Nkuekam et al. 2008a, 2010, 2011, 2012a; Grobbelaar et al. 2009a, 2010) and mating compatibility tests (Brasier and Mehrotra 1995; Uzunovic 2000; Chung et al. 2006; Grobbelaar et al. 2010) are known to be useful for recognizing phylogenetic species in the *O. piceae* complex. Thus, these methods in

addition to the more precisely morphological comparisons were considered to be essential to determine taxonomic status of the *O. piceae* complex related to Japanese larch.

1-3. Objectives of this study

Since phylogenetic relationships and taxonomic treatments of the populations mentioned above were unknown, because of lack of molecular phylogenetic analyses and precisely morphological studies in the *O. piceae* complex in Japan, the purpose of this study was to establish a new taxonomic system of the *O. piceae* complex, and to clarify taxonomy of the *O. piceae* complex from Japanese larch based on molecular phylogenetic analyses, morphological study and mating compatibility tests. In the chapter 2, molecular phylogenetic analyses of *O. piceae* complex including not only populations on Japanese larch but also those on other conifers in Japan, Canada and Europe, were conducted to clarify their phylogenetic relationship. From the chapters 3 to 5, precise morphological observation and mating compatibility tests on the distinct population detected by the phylogenetic studies in the chapter 2 were conducted to determine taxonomic status of each of the population.

Chapter 2. Molecular phylogenetic analyses of the *Ophiostoma piceae* complex

To clarify the phylogenetic relationship among *Ophiostoma piceae* complex associated with bark beetles infesting Japanese larch as well as other conifers in Japan, Canada and Europe, molecular phylogenetic analyses of the fungi using ITS nrDNA region, the partial β-tubulin and EF-1α genes were conducted.

2-1. Materials and Methods

2-1-1. Isolates of fungi

A total of 23 isolates of *Ophiostoma piceae* complex from Japanese larch were selected among the isolates deposited in culture collection of the Laboratory of Plant Parasitic Mycology, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan. Those isolates were isolated from bark beetle and beetle galleries, i.e., *Ips subelongatus*, *Doryocoetes* spp., *Polygraphus* spp. and *Cryphalus* sp. collected in Nagano and Tochigi prefectures in central Honshu and Iwate prefecture, northern Honshu of Japan.

A total of 15 isolates of *O. piceae* from *Pinus* and *Picea* in Japan, Canada, and Europe were also used for the phylogenetic analyses. In addition, four isolates of *O. subalpinum* and one Japanese isolate of *O. canum* and three isolates of *O. quercus* were added to the analyses. Details of isolates used in the phylogenetic analyses were summarized in Table 2.1. Reference sequences obtained from NCBI GenBank were used for the analyses. Detailed information was shown in Table 2.2.

2-1-2. DNA extraction, PCR amplification and sequencing

Isolates were cultured on 1% Genmai flake agar [1% GFA: 10 g Kellogg's Genmai

flakes (Corporate of Kellogg, Japan), 18 g agar, 1000 ml distilled water] for 2 wk. DNA was extracted using a modified version of the extraction method described by Linnakoski et al. (2008). Approximately 50 mg of mycelium were transferred to sterilized Eppendorf tubes, suspended in 200 ml of DNA extraction buffer [10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% sodium dodecyl sulphate (SDS), 0.01% Proteinase K], ground with a disposable pestle, and incubated for 10 min and centrifuged at 14 000 rev. / min for 5 min. The supernatant was transferred to new Eppendorf tubes and precipitated using Ethachinmate (Wako Pharmaceutical, Tokyo, Japan).

To amplify ITS nrDNA region, primer pair ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) was used. Amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA), with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min 30 s. The reaction was completed by a 10 min extension at 72 °C. To amplify the partial sequence of β-tubulin, primer pair T10 (O'Donnell and Cigelnik 1997) and BT12 (Kim et al. 2003) was used. Amplification was performed with an initial denaturation at 94 °C for 4 min, followed by 9 cycles of 94 °C for 30 s, 47°C to 56 °C for 30 s, and 72 °C for 1 min 30 s, and 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min 30 s. The reaction was completed by a 10 min extension at 72 °C. To amplify the partial sequence of EF-1α, primer pair EF1F and EF2R (Jacobs et al. 2004) was used. PCR was performed as described by Linnakoski et al. (2012). Amplification was performed with an initial denaturation at 95 °C for 5 min, followed by 9 cycles of 95 °C for 35 s, 60°C to 66 °C for 55 s, and 72 °C for 1 min 30 s, and 35 cycles of 95 °C for 35 s, 56 °C for 30 s, and 72 °C for 1 min 30 s. The reaction was completed by a 10 min extension at 72 °C.

PCR products were purified using a Wizard® SV gel and a PCR Clean-up Kit (Promega, Madison, WI, USA). The purified PCR products were directly sequenced using a BigDye™ Terminator Cycle Sequencing kit v. 3.1 (Applied Biosystems) following the manufacturer's instructions and analyzed on an ABI PRISM® 3130 DNA Analyzer (Applied Biosystems, USA). Sequences were assembled with ATGC ver. 7.0.0 software (Genetyx, Tokyo, Japan) and deposited in GenBank.

2-1-3. Phylogenetic analyses

Sequences were aligned using the online version of MAFFT 7 with the G-INS-i option (http://mafft.cbrc.jp/alignment/server/) (Katoh and Standly 2013).

Phylogenetic analyses were inferred with maximum parsimony (MP) using PAUP v. 4.0b10 (Swofford 2003), maximum likelihood (ML) using GARLI v. 0.951 (Zwickl 2006), and Bayesian Markov chain Monte Carlo (MCMC) using MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003). MP analysis was performed using the heuristic search option with 1000 replications and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. All characters were equally weighted and gaps were treated as missing data. ML analysis was conducted using the GTR+I+Γ_4 model consisting of the GTR substitution process (Lanave et al. 1984; Tavaré 1986) with a four category discrete approximation to gamma-distributed rate heterogeneity (Yang 1994) and an inferred proportion of invariable sites. The tree topology with the highest likelihood was inferred from 10 independent runs from random starting trees. The "stopgen" parameters were set to 50,000,000 and other parameters were set to default values. Bootstrap analysis was performed using 1000 replications with the same parameters as the initial tree search. In Bayesian inference analysis, the best-fit substitution models for different datasets were estimated using MrModeltest v. 2.3 (Nylander 2004) based on the implementation of the Akaike information criterion

(AIC). Four Markov chains were run twice from random starting trees for 10,000,000 generations, and trees were sampled every 500 generations. The first 25% of all generations were discarded as burn-in and a majority rule consensus tree of all remaining trees was calculated to determine the posterior probabilities for the individual branches. Outgroup used were *Sporothrix abietina (*NCBI GenBank accession number: AF484453) and *S. stenoceras* (AF1484462) for the ITS nrDNA phylogenetic tree. Because the *O. ulmi* complex has been inferred from the phylogenetic analyses of ITS nrDNA and LSU gene regions (De Beer and Wingfield 2013), and distinct from other *Ophiostoma s. str.*., therefore, outgroup used were *O. ulmi* (EU977489) and *O. novo-ulmi* (AY305712) for the phylogenetic analyses of the partial β-tubulin. Outgroup used was *O. ulmi* (HQ292093) for the EF-1α phylogeny. Outgroup used was *O. ulmi* also for the concatenated ITS rDNA, β-tubulin and EF-1α phylogeny. Reference sequences were obtained from NCBI GenBank (Table 2.2).

Table 2.1. Isolates of the *Ophiostoma piceae* complex used for phylogenetic analyses and morphological study.

Species/Groups	Isolate no. (Other no. [a])	Origin	Locality of collection	Collector	Mating type	Amplified sequences		
						ITS	β-tubulin	EF-1α
Group C	YCC-588 (NBRC 105442)	*Polygraphus kisoensis* in *Larix kaempferi*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	○	○	○
	YCC-589 (NBRC 111723)	*P. kisoensis* in *L. kaempferi*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	○	○	○
	YCC-839	*P. kisoensis* in *L. kaempferi*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	○	○	○
	YCC-840	*Cryphalus* sp. in *Picea koyamae*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed		○	○
	YCC-841	*Cryphalus* sp. in *P. koyamae*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed		○	○
	YCC-640	*Polygraphus horyurensis* in *Pinus banksiana*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	○	○	○
O. breviusculum	YCC-326	*Ips subelongatus* in *L. kaempferi*	Kawakami, Nagano, Japan	Yamaoka Y.	Mixed	○	○	○
	YCC-327	*I. subelongatus* in *L. kaempferi*	Kawakami, Nagano, Japan	Yamaoka Y.	Mixed	♦	♦	○
	YCC-494 (JCM11980)	*Dryocoetes baikalicus* in *L. kaempferi*	Nikko, Tochigi, Japan	Yamaoka Y.	Mixed	♦	♦	○
Group A	YCC-492	*I. subelongatus* in *L. kaempferi*	Yumihari Pass, Nikko, Tochigi, Japan	Yamaoka Y.	Mixed	○	○	○
	YCC-493	*I. subelongatus* in *L. kaempferi*	Yumihari Pass, Nikko, Tochigi, Japan	Yamaoka Y.	Mixed	○	○	○
Group B	YCC-586	*D. pini* in *L. kaempferi*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	○	○	○
	YCC-587	*D. pini* in *L. kaempferi*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	○	○	○
Group D	YCC-322	*I. subelongatus* in *L. kaempferi*	Kawakami, Nagano, Japan	Yamaoka Y.	Mixed	○	○	○
	YCC-384	*I. subelongatus* in *L. kaempferi*	Sumida-machi, Iwate, Japan	Yamaoka Y.	Mixed	○	○	○
	YCC-301	*I. subelongatus* in *L. kaempferi*	Kawakami, Nagano, Japan	Yamaoka Y.	Mixed	○	○	-
	YCC-345	*I. subelongatus* in *L. kaempferi*	Near Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	-	○	-
	YCC-385	*I. subelongatus* in *L. kaempferi*	Sumida-machi, Iwate, Japan	Yamaoka Y.	Mixed	○	○	○
	YCC-443	*D. hectographus* in *L. kaempferi*	Nikko, Tochigi, Japan	Yamaoka Y.	Mixed	-	○	○
	YCC-595	*Dryocoetes pini* in *L. kaempferi*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	-	-	○
	YCC-596	*D. hectographus* in *L. kaempferi*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	-	○	○
	YCC-832	*D. autographus* in *L. kaempferi*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	-	○	○
	YCC-833	*D. pini* in *L. kaempferi*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	-	-	○
O. piceae	YCC-718	*Hylurgops transbaicalicus* in *Pinus parviflora* var. *pentaphylla*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	○	○	○

Table 2.1. Isolates of the *Ophiostoma piceae* complex used for phylogenetic analyses and morphological study (**Continued**).

Species/Groups	Isolate no. (Other no. [a])	Origin	Locality of collection	Collector	Mating type	Amplified sequences ITS	β-tubulin	EF-1α
O. piceae	YCC-720	*H. transbaicalicus* in *P. parviflora* var. *pentaphylla*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	-	-	○
	YCC-731	*H. transbaicalicus* in *P. parviflora* var. *pentaphylla*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	○	-	○
	YCC-563	*D. autographus* in *Pin. strobus*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	○	○	○
	YCC-562	*H. transbaicalicus* in *Pin. strobus*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	-	○	-
	YCC-701	*H. transbaicalicus* in *Picea koyamae*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	○	○	○
	YCC-702	*H. transbaicalicus* in *Pic. koyamae*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	-	○	○
	YCC-637	*Tomicus piniperda* in *Pin. banksiana*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	○	○	○
	YCC-732	*D. autographus in Pic. glauca*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	-	○	○
	YCC-700	*H. transbaicalicus* in *Pic. koyamae*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	-	○	○
	AU100-1	*Pic. mariana*	Saskatchewan, Canada		A	-	○	○
	H2181 (C967, CMW7648)	*Pic. sitchensis*	UK		A	-	○	○
	AU135-1	*Pic. glauca*	Prince George, Canada		B	○	○	○
	H2134	*Pin. mgra*	Norfolk, UK		B	○	○	○
	H2154	*Picea* sp.	Poland		A	-	○	○
O. subalpinum	YCC-408 (JCM11716)	*Cryphalus montanus* in *Abies mariesii*	Border between Tochigi and Gunma, Japan	Yamaoka Y.	Mixed	-	-	○
	YCC-410	*C. montanus* in *A. mariesii*	Border between Tochigi and Gunma, Japan	Yamaoka Y.	Mixed	-	-	-
	YCC-580	*C. montanus* in *A. mariesii*	Border between Tochigi and Gunma, Japan	Yamaoka Y.	Mixed	-	○	○
	YCC-411 (JCM11717)	*C. montanus* in *A. mariesi*	Nikko, Tochigi, Japan	Yamaoka Y.	Mixed	-	○	-
O. canum	YCC-685	*Polygraphus* sp. in *Pin. densiflora*	Sugadaira, Nagano, Japan	Yamaoka Y.	Mixed	-	○	-
O. floccosum	GR10 (C1013)	*Pin. nigra* var. *maritima*	Thetford Forest, UK		B	○	-	-
O. quercus	H1039 (CBS102353, C970)	*Quercus* sp.	Surrey, UK		A	-	-	○
	H2190	*Quercus* sp.				-	○	○
	YCC-659	*Populus nigra*	Sugadaira, Nagano, Japan			-	-	○

[a] AU, A. Uzunovic's personal collection, FP Innovations-Forintek Division, Vancouver, Canada. C: T. C. Harrington's personal collection, Department of Plant Pathology and Microbiology, Iowa State University, Ames, USA. CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. CMW: Cultures of M. J. Wingfield, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa. JCM: Japan Collection of Microorganisms, RIKEN BioResource Center, Tsukuba, Japan. YCC: Cultures of Y. Yamaoka, Laboratory of Plant Parasitic Mycology, Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan. ○ Sequence was successfully amplified from the isolates.

Table 2.2. Details of reference sequences of the *Ophiostoma piceae* complex retrieved from GenBank database.

Species	Isolate no. [a]	Locality of collection	Host	GenBank accession no.			References
				ITS	β-tubulin	EF-1α	
O. piceae	H2154	Poland	*Picea* sp.	AF081131	–	–	Kim et al. (1999)
	AU100-1	Saskatchewan, Canada	*Pic. mariana*	AF081129	–	–	Kim et al. (1999)
	C1087 (CBS 108.21, CMW 25034) ex-holotype	Germany	–	AF198226	–	KU184398	Harrington et al. (2001)
		Germany	–	KU184441			Yin et al. (2006)
	H2009 (C968, CMW 7649)	UK	*Pin. sylvestris*	–	–	KF899886	Kim et al. (1999)
	CMW 13243 (CBS 102356)	USA	*Pin. menziesii*	–	–	KU184397	Yin et al. (2016)
	CMW 13239 (CBS 819.85)	Canada	*Betula papyrifera*	KU184438	–	–	Yin et al. (2016)
O. canum	C1088 (CBS 133.51)	Sweden	*Pin. sylvestris*	HM031489	–	–	Linnakoski et al. (2010)
	CBS118668	Germany		–	JQ886729	–	–
	AU30	Norway	*Pin. sylvestris*	–	EU977485	–	Massoumi Alamouti et al. (2009)
	CMW 29495 (CBS 124499)	Norway	*Betula pendula*	KU184424	–	–	Yin et al. (2016)
O. subalpinum	YCC-408	Border between Tochigi and Gunma, Japan	*Abies mariesii*	AB200424	–	–	Chung et al. (2006)
	YCC-410	Border between Tochigi and Gunma, Japan	*A. mariesii*	AB200425	AB200430	–	Chung et al. (2006)
	MAFF 410924	Yamanashi, Japan	*A. mariesii*	AB096211	–	–	Masuya et al. (2003)
	MAFF 410923	Yamanashi, Japan	*A. mariesii*	AB096210	–	–	Masuya et al. (2003)
O. breviusculum	YCC-494	Nikko, Tochigi, Japan	*Larix kaempferi*	AB200422	–	–	Chung et al. (2006)
	YCC-327	Kawakami, Nagano, Japan	*L. kaempferi*	AB200420	AB200426	–	Chung et al. (2006)
O. rachisporum	CMW 23271	Ilomantsi, Finland	*Pin. sylvestris*	–	HM031515	KU184407	Linnakoski et al. (2010); Yin et al. (2016)
	CMW 23273	Punkaharju, Finland	*Pin. sylvestris*	KU184449	–	–	Yin et al. (2016)
	CMW 28021	Russia	*Pin. sylvestris*	–	HM031512	–	Linnakoski et al. (2010)
	CMW 23272 (CBS 128119)	Ilomantsi, Finland	*Pin. sylvestris*	KU184448	–	–	Yin et al. (2016)
O. setosum	CMW 37441 (CBS 102358)	USA	*Pseudotsuga menziesii*	KU184425	−	−	Yin et al. (2016)
	CMW 27833	Canada	*Tsuga heterophylla*	KU184451	−	−	Yin et al. (2016)
	CMW 27834	Canada	*T. heterophylla*	KU184452	−	−	Yin et al. (2016)
O. floccosum	CMW12623	Australia	*Pin. sylvestris*	KU184428	−	−	Yin et al. (2016)
O. novo-ulmi	C1185 (CBS298.87, WCS637)	Russia	*Ulmus* spp	AF198235	−	−	Harrington et al. (2001)
	C1182 (CBS102.63, IMI101223, JCM9303)	Netherland	Ulmus spp	AF198232	−	−	Harrington et al. (2001)

Table 2.2. Details of reference sequences of the *Ophiostoma piceae* complex retrieved from GenBank database (**Continued**).

Species	Isolate no. [a]	Locality of collection	Host	GenBank accession no.			References
				ITS	β-tubulin	EF-1α	
O. himal-ulmi	C1183 (CBS374.67, ATCC36176, ATCC36204)	India	*Ulmus* spp	AF198233	–	–	Harrington et al. (2001)
O. quercus	C969 (CBS105352, H1042)	UK	*Quercus* sp.	AF198238	JQ886713	–	Harrington et al. (2001) Hyun et al. (2012)
	C970 (CBS105353, H1039)	UK	*Quercus* sp.	AF198239	JQ886709	–	Harrington et al. (2001); Hyun et al. (2012)
O. flexuosum	CMW907	Norway	*Pic. abies*	KU184427	DQ296090	KU184384	Yin et al. (2016); Zipfel et al. (2006)
	FAE1D-4-11-Of	Canada	*Pic. glauca*		FJ269204		–
O. distortum	CMW 40668 (CBS 429.82)	USA	*A. concolor*	KU184426	–	–	Yin et al. (2016)
O. brunneum	CMW 1027 (CBS 161.61)	USA	*A. lasiocarpa*	KU184423	–	KU184380	Yin et al. (2016)
O. ssiori	MAFF 410973	Morioka, Iwate, Japan	*Prunus* sp.	AB096209	–	–	Masuya et al. (2003)
O. australiae	CMW 6606	Australia	*Acacia mearnsii*	EF408603	–	–	Kamgan Nkuekam et al. (2008)
O. catonianum	C1084 (CBS 263.35)	Italy	*Pyrus communis*	AF198243	–	–	Harrington et al. (2001)
	CMW 18966	Norway	*B. pubescens*	EF408593	–	–	Kamgan Nkuekam et al. (2010)
O. ips	C327	USA	–	AF198244	–	–	Harrington et al. (2001)
O. nitidum	CMW 38905 (CBS 136526)	China	*Pic. crassifolia*	KU184436	–	KU184393	Yin et al. (2016)
	CMW 38907 (CBS 136525)	China	*Pic. crassifolia*	KU184437	–	–	Yin et al. (2016)
O. micans	CMW 38903 (CBS 136523)	China	*Pic. crassifolia*	KU184432	–	KU184389	Yin et al. (2016)
	CMW 38909 (CBS 136524)	China	*Pic. crassifolia*	KU184433	KU184304	–	Yin et al. (2016)
O. qinghaiense	CMW 38902 (CBS 136521)	China	*Pic. crassifolia*	KU184445	–	–	Yin et al. (2016)
	CMW 38904 (CBS 136522)	China	*Pic. crassifolia*	KU184446	–	–	Yin et al. (2016)
	CMW 38906	China	*Pic. crassifolia*	–	KU184318	KU184404	Yin et al. (2016)
O. nikkoense	CMW 7193 (JCM 11728)	Japan	*A. mariesii*	KU184434	–	–	Yin et al. (2016)
	CMW 7194 (JCM 11729)	Japan	*A. homolepis*	KU184435	–	–	Yin et al. (2016)
O. arduennense	CMW 40266 (MUCL 44866)	Belgium	*Fagus sylvatica*	KU184419	–	–	Yin et al. (2016)
O. torulosum	CMW 10574	Austria	*F. sylvatica*	KU184458	–	–	Yin et al. (2016)
	CMW 40670 (CBS 770.71)	Germany	*F. sylvatica*	KU184457	–	–	Yin et al. (2016)
O. araucariae	CMW 40665 (CBS 114. 68)	Chile	*Araucaria araucana*	KU184418	–	–	Yin et al. (2016)
O. denticiliatum	CMW 29493	Norway	*Betula* sp.	FJ804490	–	–	Linnakoski et al. (2009)
O. undulatum	CMW 19396	Australia	*Eucalyptus grandis*	GU797218	–	–	Kamgan Nkuekam et al. (2011)
O. tasmaniense	CMW 29088	Australia	*E. nitens*	GU797211	–	–	Kamgan Nkuekam et al. (2011)
O. tsotsi	CMW 15239	Malawi	*E. grandis*	FJ441287	–	–	Grobbelaar et al. (2010)
O. bacillisporum	MUCL 45378	Belgium	*F. sylvatica*	AY573258	–	–	Carlier et al. (2006)
O. tetropii	CBS 428.94	Austria	*Pic. abies*	AY934524	–	–	Villarreal et al. (2005)

Table 2.2. Details of reference sequences of the *Ophiostoma piceae* complex retrieved from GenBank database (**Continued**).

Species	Isolate no. [a]	Locality of collection	Host	GenBank accession no.			References
				ITS	β-tubulin	EF-1α	
O. kryptum	DAOM 229701	Austria	*Pic. abies*	AY304436	–	–	Jacobs and Kirisits (2003)
O. allantosporum	CBS 185.86	USA	*Pin. resinosa*	AY934506	–	–	Villarreal et al. (2005)
O. minus	AU58.4	Canada	*Pin. contorta*	AF234834	–	–	Schroeder et al. (2001)
O. piliferum	CBS 129.32	Europe	*Pin. sylvestris*	AF221070	–	–	Schroeder et al. (2001)
O. triangulosporum	DSM 4934	Brazil	*Ara. araucana*	AY934525	–	–	Villarreal et al. (2005)
O. karelicum	CMW 23099	Russia	*B. pendula*	EU443762	–	–	Linnakoski et al. (2008)
O. longiconidiatum	CMW 17574	South Africa	*Terminalia sericea*	EF408558	–	–	Kamgan Nkuekam et al. (2008)
O. conicola	CBS 127.89	Mexico	*Pin. cembroides*	AY924384	–	–	Villarreal et al. (2005)
O. subannulatum	CBS 188.86	USA	*Pinus sp.*	AY934522	–	–	Villarreal et al. (2005)
O. pluriannulatum	MUCL 18372	USA	*Conifer*	AY934517	–	–	Villarreal et al. (2005)
O. sparsiannulatum	CMW 17231	USA	*Pin. taeda*	FJ906817	–	–	Zanzot et al. (2010)
O. multiannulatum	MUCL 19062	USA	*Pinus sp.*	AY934512	–	–	Villarreal et al. (2005)
O. ainoae	CMW 1037 (CBS 205.83)	Norway	*Pic. abies*	KU184416	–	–	Yin et al. (2016)
O. poligraphi	CBS 128299 (CMW 23123)	Russia	*Pic. abies*	KU184417	–	–	Yin et al. (2016)
O. shangrilae	CMW 38898 (CBS 136518)	China	*Pic. crassifolia*	KU184443	–	–	Yin et al. (2016)
	CMW 38899 (CBS 136517)	China	*Pic. crassifolia*	KU184444	–	–	Yin et al. (2016)
	CMW 38900 (CBS 136520)	China	*Pic. crassifolia*	KU184453	–	–	Yin et al. (2016)
	CMW 38901 (CBS 136519)	China	*Pic. crassifolia*	KU184454	–	–	Yin et al. (2016)
O. brunneociliatum	CMW 5212	Scotland	*Larix sp.*	KU184422	–	–	Yin et al. (2016)
	CBS 117571	Scotland	*L. decidua*	KU184421	–	–	Yin et al. (2016)
O. tapionis	CMW 23265 (CBS 128120)	Finland	*Pic. abies*	KU184455	–	–	Yin et al. (2016)
	CMW 23269 (CBS 128121)	Russia	*Pin. sylvestris*	KU184456	–	–	Yin et al. (2016)
O. japonicum	YCC-99	Japan	*L. kaempferi*	GU134169	–	–	–
O. montium	CMW 13221	USA	*Pin. ponderosa*	AY546711	–	–	Zhou et al. (2004a)
O. fuscum	CMW 23196	Finland	*Pin. abies*	HM031504	–	–	Linnakoski et al. (2010)
O. bicolor	CBS 492.77	USA	*Pic. glauca*	DQ268604	–	–	Massoumi Alamouti et al. (2007)
O. pulrinisporum	CMW 9022	Mexico	*Pin. pseudostrobus*	AY546714	–	–	Zhou et al. (2004)
O. adjuncti	CMW 135	USA	*Pin. ponderosa*	AY546696	–	–	Zhou et al. (2004)
Sporothrix abietina	CBS 125.89	Mexico	*Abies vejari*	AF484453	–	–	De Beer et al. (2003a)
S. stenoceras	CBS 237.32	Norway	Pine pulp	AF484462	–	–	De Beer et al. (2003a)

[a] YCC: Cultures of Yuichi Yamaoka, Culture collection of the Laboratory of Plant Parasitic Mycology, Graduate School of Life and Environmental Sciences. University of Tsukuba, Tsukuba, Japan; CBS: Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands. CMW: Cultures of

Michael J. Wingfield, Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. JCM: Japan Collection of Microorganisms, Tsukuba, Japan.
− Information is unavailable.

2-2. Results

2-3. Discussion

Results and discussion are not disclosed yet.

Chapter 3. Morphological study and mating compatibility tests of Group C

Phylogenetic analyses on the ITS nrDNA region, β-tubulin, EF-1α genes and concatenated ITS nrDNA, β-tubulin, EF-1α genes conducted in the Chapter 2 showed that Group C was monophyletic and distinguishable from other *O. piceae* complex species. In this chapter, morphological studies and mating compatibility tests of Group C were conducted to determine the taxonomic status of this group.

3-1. Materials and Methods

3-1-1. Isolates used in morphological and cultural study

Because of close phylogenetic relationships with Group C, four conifer-inhabiting species, *O. piceae*, *O. breviusculum*, *O. canum* and *O. subalpinum* were used for morphological comparison.

Five isolates of Group C, YCC-588, YCC-589, YCC-640, YCC-839 and YCC-786 and five Japanese isolates of *O. piceae*, YCC-563, YCC-637, YCC-701, YCC-718 and YCC-731 were used for morphological observation of synnematous characteristics. As for *O. breviusculum*, three isolates, YCC-326, YCC-327, YCC-494 and two mixed mating cultures between YCC-519 and YCC-522 and YCC-519 and YCC-532, were used.

The two isolates, YCC-588 and YCC-589, were used for morphological observation of sexual morphs because of the absence of perithecia and ascospores in other isolates of the present fungus. These two isolates originated from masses of ascospores. For comparison, two isolates each of *O. piceae*, *O. breviusculum*, and *O. subalpinum* were examined. Moreover, the descriptions of *O. canum* by Hunt (1956)

and Mathiesen (1951) were used.

The three Group C isolates, YCC-588, YCC-589, and YCC-640, were used for growth assays at 25 °C and 32 °C, which was used to distinguish between *O. piceae* and *O. querci* (Brasier and Stephens 1993). An agar disk 4 mm diam cut from the edge of an actively growing colony was placed at the center of a 1% GFA plate and incubated at 25 °C and 32 °C in the dark for 1 wk. Triplicate plates were prepared for each isolate. The diameter of each colony was measured twice at right angles and an average was calculated.

3-1-2. Methods of morphological and cultural study

Living cultures used for morphological studies were grown on 1% GFA plates at 16 °C in the dark. After 2-mo incubation, small pieces of autoclaved bark of Japanese larch (about 2 cm × 5 mm × 3 mm) were added to the cultures to stimulate production of perithecia and synnemata. Perithecia and synnemata produced on the bark were randomly removed and mounted in a mixture of Melzer's reagent and polyvinyl alcohol-lactic acid-glycerol (PVLG) on glass slides and then observed under an Olympus BHS-Nomarski interference contrast microscope (Olympus Optical, Tokyo, Japan). Ascospores and conidia on synnematous/mononematous conidiophores were mounted in 1% lacto-fucsin on glass slides and then examined.

A total of 30 measurements for each morphologically relevant structure were taken. Dimensions of the perithecia, ascospores, synnemata, and conidia on synnematous/mononematous conidiophores were measured using Wraycam software (Wraymer, Osaka, Japan). The measured synnema data were tested for one-way analysis of variance (ANOVA) and Duncan's multiple range tests were applied for comparing mean values using the software package SPSS (SPSS Japan, Tokyo,

Japan).

The cultures of the present fungus have been deposited in the Japan Collection of Microorganisms (JCM), RIKEN BioResource Center, Tsukuba, Japan, and in the Biological Resource Center National Institute of Technology and Evaluation (NBRC), Kisarazu, Japan. Dried specimens of the cultures of the present fungus used for morphological studies have been deposited in the Herbarium of the Life and Environmental Sciences, University of Tsukuba (TSH). Holotype specimens were deposited in the mycological herbarium of the National Museum of Nature and Science, Tsukuba, Japan (TNS).

3-1-3. Isolates used in mating compatibility tests

Four representative single ascospore isolates, YCC-785 and YCC-786 (mating type "+"), and YCC-782 and YCC-783 (mating type "–"), were used for mating compatibility tests. These four isolates were selected from among 10 single ascospore isolates obtained by the same method as used by Chung et al. (2006) from the isolate YCC-589, which originated from a mass of ascospores. To determine the mating type of each isolate, mating compatibility tests were conducted in all of the possible combinations among the 10 isolates. These four single ascospore isolates were paired with tester isolates of *O. piceae*, *O. breviusculum*, and *O. subalpinum* collected in Japan, and isolates of *O. piceae* from other countries. Methods for mating compatibility tests were the same as described by Chung et al. (2006).

Production of perithecia on 1% GFA medium was observed at intervals of 2 wk and scored after more than 2 mo of incubation as "–" (no production of perithecia), "+" (abundant production of perithecia with ascospores), or "±" (minor production of

perithecia with or without ascospores).

To confirm viability of single ascospore F1 progeny isolates and variation of the colonies originating from a cross between isolate YCC-785 and *O. piceae* AU135-1, single ascospore isolation was conducted by the same method as used by Chung et al. (2006). Mating compatibility tests among the single ascospore F1 progeny isolates established from the crosses between isolate YCC-785 and AU135-1 were conducted in all possible combinations. In addition, morphology of a small number of perithecia originating from a cross between isolate YCC-783 and *O. piceae* H2181 were examined using the methods described above.

3-2. Results

3-2-1. Morphological and cultural study

Group C produced black perithecia with a long neck (Fig. 3.1A) and ostiolar hyphae at the tip of the neck (Fig. 3.1B). Ascospores were orange-section shaped or reniform (Fig. 3.1D). Asexual morphs were both pesotum-like (Fig 3.1C) and sporothrix-like (Fig. 3.1F). Synnemata erect, light brown at the base and the apex, becoming dark brown to black toward the middle (Fig 3.1C). Conidia on pesotum-like states were oblong, clavate, or obovoid (Fig. 3.1E). Conidia on sporothrix-like states were oblong, clavate, or obovoid (Fig. 3.1F). A detailed description of the morphology, including measurements in the sexual morph and the asexual morphs, is provided in the Taxonomy section.

The present fungus was characterized based on the distinct color of the synnema stipe, which was brown at the base, gradually becoming black at the middle, and light brown at the apex (Table 3.1). This unique characteristic distinguished the present

fungus from *O. piceae* and *O. breviusculum*, both of which had dark brown to black bases, brown to light brown middles, and light brown to white apices. Furthermore, the synnemata of the present fungus were narrower at the middle than at the base. This characteristic was numerically demonstrated based on the ratio of synnema base width to middle width (Table 3.4). Synnemata of the present fungus were larger (1.20−3.92; mean values ranged from 1.90−2.12) than those of *O. breviusculum* (0.96−2.39; mean values ranged from 1.52−1.68). The mean length in the present fungus (mean values ranged from 295.2−441.8 μm) was longer than that of *O. breviusculum* (mean values ranged from 206.8−299.8 μm).

The present fungus grew at 25 °C, but exhibited no growth at 32 °C. The average diameter of colonies of the three isolates was 45.2 mm after 1 wk incubation at 25 °C. Colonies on 1% GFA medium were white at first, later becoming light brown, without forming concentric rings on the surface of the colonies after 1 mo of incubation at 25 °C in the dark (Fig. 3.2).

3-2-2. Mating compatibility tests

When strains of "+" and "−" mating types were paired on a plate with 1% GFA, fertile perithecia with ascospore masses were abundantly produced after approximately 2 wk of incubation at 16 °C (Table 3.5).

The representative isolates of the present fungus did not produce perithecia by mating with tester isolates of *O. piceae*, *O. breviusculum*, and *O. subalpinum* after 2 wk of incubation (Table 3.5). However, one isolate (YCC-785; "+" mating type) produced a few perithecia with *O. piceae* AU135-1 ("B" mating type) after 2 mo of incubation. Moreover, one isolate (YCC-783; "−" mating type) produced a few fertile perithecia with one isolate each of *O. breviusculum* (YCC-519; "A" mating type) and

O. subalpinum (YCC-581; “A” mating type) after 2 mo of incubation. In addition, a few perithecia were produced in a cross between one isolate (YCC-785; “+” mating type) and *O. piceae* (H2181 “A” mating type). Four single ascospore isolates of the present fungus derived from YCC-588 did not produce perithecia by mating with tester isolates of *O. piceae* after 2 wk of incubation (Table 3.5). A few sterile perithecia were produced after 2 mo of incubation.

Perithecia from mating between isolate YCC-783 and *O. piceae* (H2181) have a narrower perithecial base (range commonly 29−50 μm diam at base) and shorter perithecial neck (range commonly 134−303 μm long) than those of the parent species. Perithecia produced from the cross between isolate YCC-783 and *O. breviusculum* YCC-519 and the cross between isolate YCC-783 and *O. subalpinum* YCC-581 were morphologically similar to *O. breviusculum* and *O. subalpinum*, respectively.

Single ascospore isolates (F1 progeny) established from a single perithecium, which originated from the cross between isolate YCC-785 and *O. piceae* (AU135-1), exhibited abnormal colony characteristics not found in either species (Fig. 3.2). These colonies varied in growth rate, color from white to dark brown, formed concentric rings, abundant aerial mycelia, and white synnemata. Moreover, the single ascospore F1 progeny isolates obtained from the cross between isolate YCC-785 and *O. piceae* (AU135-1) did not produce perithecia by mating with each other after 2 mo of incubation at 16 °C.

Based on distinct phylogenetic relationships with the other *O. piceae* complex, unique color and shape of synnemata, and interspecific mating with closely related species, Group C was recognized as a distinct species in the *O. piceae* complex and was described as a new species in the following Taxonomy section.

3-3. Taxonomy

Ophiostoma sugadairense J. Li, Y. Yamaoka & H. Masuya, sp. nov. Fig. 3.1.

MycoBank no.: MB815565.

Diagnosis: The present fungus was distinguishable from other species in the *Ophiostoma piceae* complex by the distinct color of its synnema stipe, which was brown at the base, gradually becoming black at the middle, and light brown at the apex, and by its synnema shape, which was narrower in the middle than at the base.

Type: JAPAN, Nagano Pref., Ueda, Sugadaira Montane Research Center, Univ. of Tsukuba, isolated from *Polygraphus kisoensis* in *Larix kaempferi*, 11 Jul, 2006, by Y. Yamaoka (holotype, TNS-F-62120, dried culture YCC-589 grown on GFA with pieces of autoclaved bark of *L. kaempferi*; isotypes, TSH-C489, TSH-C490; ex-type culture, YCC-589, NBRC 111723).

Gene sequences ex-holotype: LC090227 (ITS nrDNA), AB934354 (β-tubulin), AB934343 (EF-1α).

Etymology: The epithet *sugadairense* refers to Sugadaira, the type locality of this species.

Perithecia superficial on the agar medium and the bark placed on the surface of the medium; basal part black, globose to subglobose (Fig. 3.1A), 95–160 (Ave. ± SD: 123 ± 17) μm diam, ornamented with short brown hyphal appendages, up to 78 μm long; necks black, straight or curved, 336–708 (480 ± 80) μm long, 23–39 (30 ± 5) μm wide at the base, 6–20 (11 ± 3) μm wide at the tip; ostiolar hyphae subhyaline to hyaline, straight, up to 35 μm long, 15–35 in number (Fig. 3.1B). Ascospores hyaline, single-celled, orange-section shaped or reniform in side view, ellipsoidal in face view, globose in end view, surrounded by a narrow hyaline sheath (Fig. 3.1D), 2.7–3.4 × 1.2–1.6 (3.0 ± 0.2 × 1.4 ± 0.1) μm including sheath, aggregating in a white droplet at

the tip of the necks. Synnemata erect, brown at the base, gradually becoming black at the middle, and light brown at the apex, pesotum type (Fig. 3.1C), 292–456 (340 ± 38) μm long including conidiogenous apparatus. The ratio of synnema base width to middle width is 1.34–2.75 (2.00 ± 0.38). Conidia on synnematous conidiophores accumulated in a slimy mass, hyaline, oblong-ellipsoidal to slightly ovate, sometimes slightly curved (Fig. 3.1E), 2.2–3.3 × 1.0–1.8 (2.6 ± 1.0 × 1.4 ± 0.6) μm. Mononematous conidiophores macronematous to micronematous, hyaline, sporothrix-type (Fig. 3.1F). Conidia on mononematous conidiophores accumulated in a slimy mass, oblong, clavate or obovoid, 3.0–8.0 × 1.4–3.0 (4.4 ± 0.8 × 2.0 ± 0.3) μm. Colonies on 1% Genmai flake agar white with aerial mycelium at first, becoming light brown after 1-mo at 25 °C. No growth at 32 °C.

Additional cultures examined: JAPAN, Nagano Pref., Ueda, Sugadaira Montane Research Center, Univ. of Tsukuba, isolated from *Polygraphus kisoensis* in *Larix kaempferi*, 11 Jul, 2006, by Y. Yamaoka, YCC-588 (NBRC 105442); JAPAN, Nagano Pref., Ueda, Sugadaira Montane Research Center, Univ. of Tsukuba, isolated from *P. kisoensis* in *L. kaempferi*, 11 Jul, 2006, by Y. Yamaoka, YCC-839; JAPAN, Nagano Pref., Ueda, Sugadaira Montane Research Center, Univ. of Tsukuba, isolated from *Cryphalus* sp. in *Picea koyamae*, 11 Jul, 2006, by Y. Yamaoka,YCC-840; JAPAN, Nagano Pref., Ueda, Sugadaira Montane Research Center, Univ. of Tsukuba, isolated from *Cryphalus* sp. in *P. koyamae*, 11 Jul, 2006, by Y. Yamaoka,YCC-841; JAPAN, Nagano Pref., Ueda, Sugadaira Montane Research Center, Univ. of Tsukuba, isolated from *Polygraphus horyurensis* in *Pinus banksiana*, 11 Jul, 2006, by Y. Yamaoka,YCC-640. Single ascospore F1 progeny isolates originating from YCC-589, YCC-782, YCC-783, YCC-785, and YCC-786.

Specimens examined: JAPAN, Nagano Pref., Ueda, Sugadaira Montane Research Center, Univ. of Tsukuba, isolated from *Polygraphus kisoensis* in *Larix kaempferi*, 11 Jul, 2006, by Y. Yamaoka (TSH-C489, isotype, dried culture YCC-589 grown on 1 % Genmai flake agar with pieces of autoclaved bark of *L. kaempferi* at 16 °C); JAPAN, Nagano Pref., Ueda, Sugadaira Montane Research Center, Univ. of Tsukuba, isolated from *P. kisoensis* in *L. kaempferi*, 11 Jul, 2006, by Y. Yamaoka (TSH-C490, isotype, dried culture YCC-589 grown on 1% Genmai flake agar with pieces of autoclaved bark of *L. kaempferi* at 16 °C); JAPAN, Nagano Pref., Ueda, Sugadaira Montane Research Center, Univ. of Tsukuba, isolated from *P. kisoensis* in *L. kaempferi*, 11 Jul, 2006, by Y. Yamaoka (TSH-C488, dried culture YCC-588 grown on 1% Genmai flake agar with pieces of autoclaved bark of *L. kaempferi* at 16 °C).

Table 3.1 Dimensions of synnemata of *Ophiostoma sugadairense* and other *O. piceae* complex.

Species	Isolate no.	Specimen no.	Synnema length (μm) [a, b]	Ratio of width of synnema base/middle [a, b]
Ophiostoma	YCC-588	TSH-C488	228.3–547.6 (295.2 ± 13.52 bc)	1.39–2.87 (1.90 ± 0.07 b)
sugadairense	YCC-589	TSH-C489	291.5–456.1 (340.4 ± 7.01 d)	1.34–2.75 (2.00 ± 0.07 bcd)
	YCC-640	TSH-C580	377.6–521.1 (441.8 ± 7.78 f)	1.52–3.24 (2.12 ± 0.08 bcd)
	YCC-839	–	230.0–434.3 (347.1 ± 8.00 d)	1.28–3.92 (2.10 ± 0.10 bcd)
	YCC-786	–	300.4–443.4 (388.2 ± 7.18 e)	1.20–3.63 (2.09 ± 0.10 bcd)
O. breviusculum	YCC-519 mated with YCC-522	TSH-C693	141.6–256.3 (206.9 ± 30.8 a)	1.00–2.27 (1.55 ± 0.06 a)
	YCC-519 mated with YCC-532	TSH-C695	158.2–329.7 (206.8 ± 38.4 a)	1.01–2.39 (1.60 ± 0.06 a)
	YCC-326	TSH-C191	187.5–291.8 (252.5 ± 32.3 b)	0.96–2.21 (1.63 ± 0.06 a)
	YCC-327	TSH-C193	233.4–381.2 (295.5 ± 42.0 bc)	1.01–1.84 (1.52 ± 0.04 a)
	YCC-494	TSH-C307	194.5–420.1 (299.8 ± 53.2 c)	1.04–2.35 (1.68 ± 0.06 a)
O. piceae	YCC-701	TSH-C614	423.5–908.3 (597.5 ± 17.20 i)	1.43–3.40 (2.20 ± 0.09 d)
	YCC-718	TSH-C704	432.0–962.0 (671.9 ± 24.40 j)	1.55–2.96 (2.22 ± 0.07 d)
	YCC-731	TSH-C705	368.7–1030.0 (536.3 ± 25.08 h)	1.81–2.53 (2.14 ± 0.04 cd)
	YCC-563	TSH-C485	328.9–754.7 (535.8 ± 19.29 h)	1.20–3.09 (1.97 ± 0.09 bc)
	YCC-637	TSH-C577	376.7–1090.7 (599.6 ± 24.76 i)	1.48–2.93 (2.06 ± 0.06 bcd)

[a] Numerical values indicate minimum – maximum (average ± standard error).

[b] Values followed by the same letter are not significantly different (P = 0.05) by Duncan's multiple range tests.

Table 3.2. Morphological comparisons of *Ophiostoma sugadairense* and closely related species.

Species	Isolate no.	Specimen no.	Perithecium [a]					Ascospore [a]		
			Base width (μm)	Neck length (μm)	Neck bottom width (μm)	Neck tip width (μm)	Ostiolar hyphae length (μm)	Shape	Length (μm)	Width (μm)
Ophiostoma sugadairense	YCC-588	TSH-C488	84–156 (116.4 ± 3.5)	368–664 (483.5 ± 16.7)	20–35 (26.8 ± 0.7)	8–18 (12.7 ± 0.5)	Up to 27	Orange-section shaped or reniform	2.4–3.6 (2.9 ± 0.03)	1.2–1.7 (1.5 ± 0.02)
	YCC-589	TSH-C489	96–163 (122.0 ± 2.9)	416–729 (584.8 ± 14.2)	20–39 (29.8 ± 0.9)	7–13 (10.6 ± 0.5)	Up to 27	Orange-section shaped or reniform	2.3–3.3 (2.9 ± 0.03)	1.1–1.6 (1.4 ± 0.02)
O. breviusculum	YCC-326	TSH-C191	59–142 (112.2 ± 3.5)	246–502 (396.6 ± 9.2)	20–41 (32.3 ± 1.1)	12–22 (16.2 ± 0.5)	Up to 26	Orange-section shaped or reniform	3.2–5.0 (4.0 ± 0.07)	1.0–1.8 (1.5 ± 0.02)
	YCC-494	TSH-C307	87–154 (113.9 ± 3.1)	276–557 (400.0 ± 13.6)	18–45 (33.1 ± 1.3)	8–13 (10.5 ± 0.2)	Up to 37	Orange-section shaped or reniform	3.0–4.0 (3.6 ± 0.04)	1.4–1.6 (1.5 ± 0.01)
O. subalpinum	YCC-408	TSH-C266	101–187 (142.7 ± 3.6)	322–1185 (694.7 ± 34.1)	19–42 (30.1 ± 1.1)	7–15 (10.1 ± 0.4)	Up to 40	Ovoid or ellipsoidal	2.5–3.4 (2.9 ± 0.03)	1.0–1.7 (1.3 ± 0.02)
	YCC-411	TSH-C286	79–168 (116.7 ± 3.5)	429–930 (647.7 ± 22.6)	20–36 (26.7 ± 0.9)	10–18 (14.2 ± 0.4)	Up to 51	Ovoid or ellipsoidal	2.5–3.7 (2.9 ± 0.04)	1.2–2.2 (1.8 ± 0.03)
O. piceae	YCC-718	TSH-C704	79–190 (134.1 ± 5.4)	379–1016 (629 ± 25.5)	17–37 (27.4 ± 0.8)	8–20 (12.4 ± 0.4)	Up to 34	Orange-section shaped or reniform	2.7–3.5 (3.0 ± 0.03)	1.1–1.7 (1.3 ± 0.02)
	YCC-731	TSH-C705	108–165 (134.6 ± 2.1)	439–1052 (686.4 ± 24.4)	20–45 (30.9 ± 1.1)	8–23 (13.9 ± 0.6)	Up to 34	Orange-section shaped or reniform	2.2–3.9 (2.9 ± 0.04)	1.1–1.4 (1.2 ± 0.02)
O. canum			50–150	Up to 1000			Average 22	Bean shaped or slightly curved (Kidney shaped)	5.0–6.0 [b] 6.5 [c]	1.5–2.5 [b] 3.0 [c]

[a] Numerical values indicate as minimum – maximum (average ± standard error).

[b] Data from Hunt (1956).

[c] Data from Mathiesen (1951).

Table 3.3 Isolates of the *Ophiostoma piceae* complex used for mating compatibility tests.

Species	Single ascospore isolates no. (Other no.) [a]	Mating type	Parental isolate no.	Origin	Locality of collection	Year
O. sugadairense	YCC-782	B	YCC-589	*Polygraphus kisoensis* in *Larix kaempferi*	Sugadaira, Nagano, Japan	2006
	YCC-783	B				
	YCC-785	A				
	YCC-786	A				
O. breviusculum	YCC-519 (JCM12500)	A	YCC-494	*Dryocoetes baikalicus* in *L. kaempferi*	Nikko, Tochigi, Japan	2001
	YCC-522 (JCM12501)	B				
O. piceae	YCC-764	A	YCC-718	*Hylurgops transbaicalicus* in *Pinus parviflora* var. *pentaphylla*	Sugadaira, Nagano, Japan	2008
	YCC-765	A				
	YCC-767	B				
	YCC-768	B				
	H2181 (C967, CMW7648)	A		*Picea sitchensis*	UK	
	AU100-1	A		*Pic. mariana*	Saskatchewan, Canada	
	AU135-1	B		*Pic. glauca*	Prince George, Canada	
O. subalpinum	YCC-584	B	YCC-408	*Cryphalus montanus* in *Abies mariesii*	Border between Tochigi and Gunma prefectures, Japan	2000
	YCC-581	A				

[a] CMW: Cultures of Michael J. Wingfield, Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. C: T. C. Harrington's personal collection, Department of Plant Pathology and Microbiology, Iowa State University, Ames, USA. JCM: Japan Collection of Microorganisms, Tsukuba, Japan. YCC: Cultures of Yuichi Yamaoka, Culture collection of the Laboratory of Plant Parasitic Mycology, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan.

Table 3.4. Distinguishing characteristics of *Ophiostoma sugadairense* and other species in the *O. piceae* complex.

Species	Color of synnema stipe	Shape of synnema conidia on conidiophores	Concentric rings in colonies [d]	Culture aroma	Growth at 32 °C for one wk (mm)
Ophiostoma sugadairense	Brown base, black middle, light brown tip	Oblong, clavate or obovoid	No	None	0
O. piceae	Black base, brown to light brown middle, light brown to white tip	Cylindrical to obovoid	Rare	None	0
O. breviusculum	Black to dark-brown base, brown to light brown middle, light brown to white tip	Oblong, clavate or obovoid	Most isolates	Weak sweet	0
O. canum [a]	Medium to dark-brown	Globose	No	None	0
O. subalpinum [a]	Olivaceous-black base, gradually becoming hyaline toward apex	Ellipsoidal or ovoid	No	None	0
O. rachisporum [b]	Hyaline, pigmented at the base	Oblong	–	–	0 at 35 °C
O. nitidum [c]	Dark brown at base becoming pale toward tip	Oblong or clavate	–	–	0 at 30 °C
O. micans [c]	Dark brown at base becoming pale toward tip	Oblong, clavate or obovoid	–	–	0 at 30 °C
O. qinghaiense [c]	Dark brown at base becoming pale toward tip	Clavate or obovoid	–	–	0 at 30 °C

[a] Data from Harrington et al. (2001).
[b] Data from Linnakoski et al. (2010).
[c] Data from Yin et al. (2016).
[d] Concentric rings of aerial mycelium, microconidiophores, and conidia in colonies.
– Unknown.

Table 3.5. Results of mating compatibility tests among *O. sugadairense*, *O. piceae*, *O. breviusculum* and *O. subalpinum*.

Species	Isolate no.	Mating type	*Ophiostoma sugadairense* [a]			
			YCC-782 B	YCC-783 B	YCC-785 A	YCC-786 A
Ophiostoma sugadairense	YCC-782	B	−	−	+	+
	YCC-783	B		−	+	+
	YCC-785	A			−	−
	YCC-786	A				−
O. piceae	AU135-1	B	−	−	± [b]	±
	H2181	A	±	±	±	−
	AU100-1	A	±	−	−	−
	YCC-767	B	−	−	±	±
	YCC-768	B	−	−	±	± [b]
	YCC-764	A	±	±	−	−
	YCC-765	A	±	±	−	−
O. breviusculum	YCC-522	B	−	−	−	−
	YCC-519	A	−	±	−	−
O. subalpinum	YCC-584	B	−	−	−	−
	YCC-581	A	−	±	−	−

[a] Production of perithecia on 1% GFA medium was observed at intervals of two wk and scored after more than two mo of incubation as "–" (no production of perithecia), "+" (abundant production of perithecia with ascospores), and "±" (minor production of perithecia with or without ascospores). [b] Single ascospore F1 progeny isolates were obtained from perithecia that originating from two crosses between *O. sugadairense* and *O. piceae*.

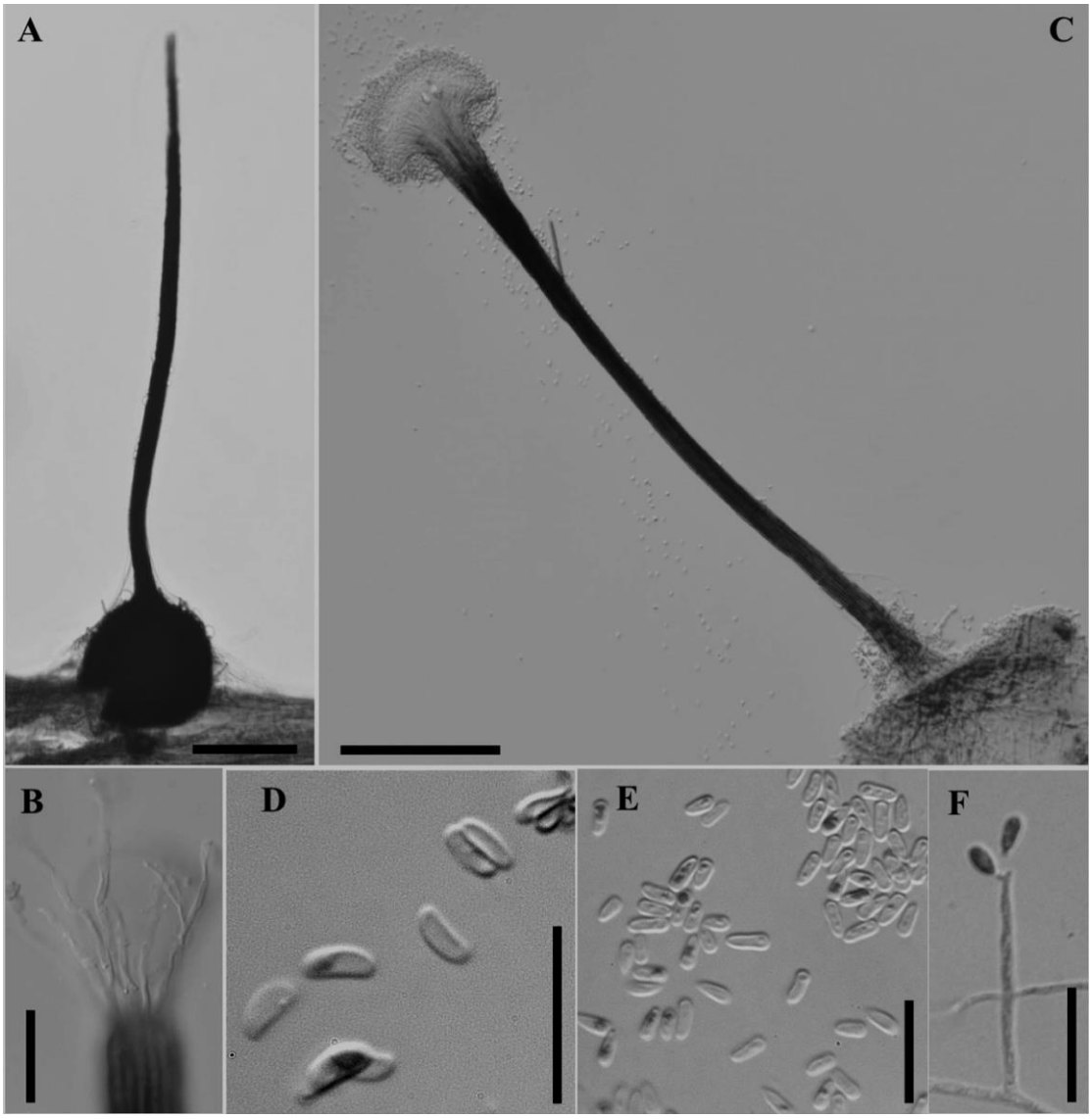

Fig. 3.1. Morphological characteristics of *Ophiostoma sugadairense* (TNS-F-62120, holotype). A: Ascocarp. B: Ostiolar hyphae on the tip of the neck. C: Synnematous conidiophore. D: Ascospores. E: Conidia on synnematous conidiophores. F: Mononematous conidiophore. *Bars*: A, C 100 μm; B, D–F 10 μm.

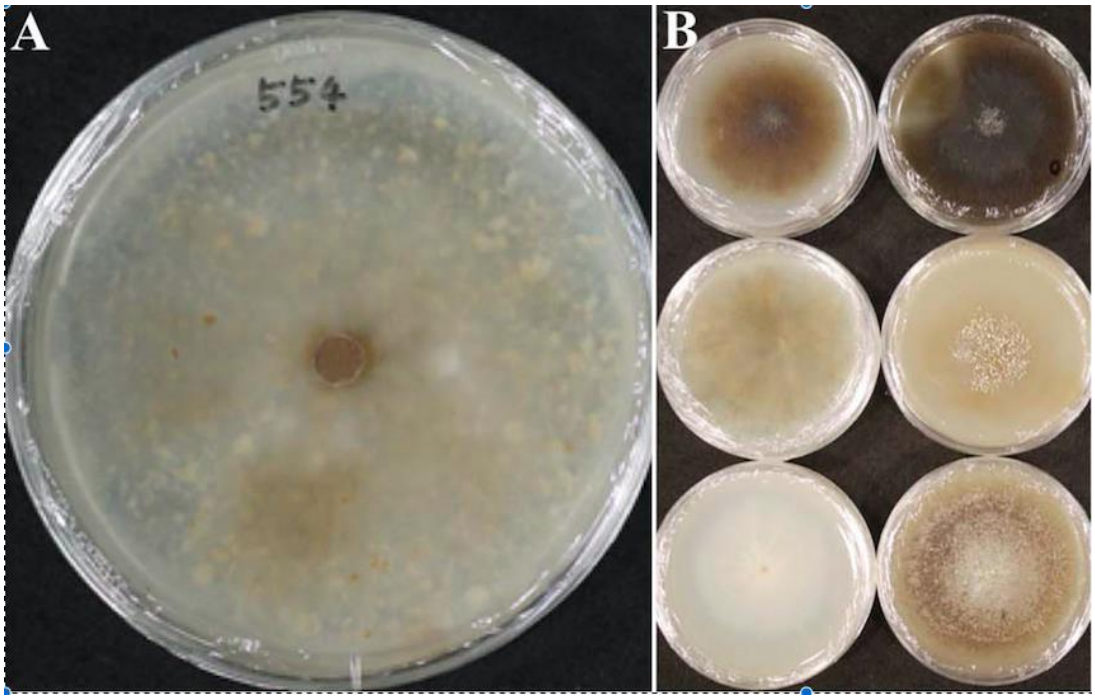

Fig. 3.2. Colonies of parental *Ophiostoma sugadairense* YCC-588 (A) and single ascospore F1 progeny isolates originating from cross between *O. sugadairense* and *O. piceae* (B) after 1 mo incubation at 16 °C in darkness. A: Parental isolate of *O. sugadairense* YCC-588. B: Abnormal colony characteristics of single ascospore F1 progeny isolates obtained from perithecium originating from 1 cross between *O. sugadairense* YCC-785 and *O. piceae* AU135-1 after 1 mo incubation at 16 °C in darkness.

3-4. Discussion

Ophiostoma sugadairense is considered a new species of the *O. piceae* complex related to conifers based on phylogenetic analyses, morphological comparisons, and mating experiments.

The nuclear protein-coding genes, β-tubulin and EF-1α genes, are considered useful in phylogenetic analyses of the *O. piceae* complex (Jacobs and Kirisits 2003; Chung et al. 2006; Kamgan Nkuekam et al. 2008a, 2010, 2011, 2012a; Linnakoski et al. 2008, 2009, 2010; Grobbelaar et al. 2009a, 2010; Yin et al. 2016), while ITS nrDNA failed to separate *O. breviusculum*, *O. brunneum*, *O. canum*, *O. flexuosum*, *O. micans*, *O. nitidum*, *O. piceae*, *O. qinghaiense*, and *O. subalpinum* (Harrington et al. 2001; Chung et al. 2006; Linnakoski et al. 2010; Yin et al. 2016). However, ITS nrDNA as well as β-tubulin and EF-1α genes were useful for distinguishing the present fungus from other species in this complex. Furthermore, variation in the intron portion of the β-tubulin gene sequences was very high and the present fungus showed a specific pattern.

Sexual morphs of the *O. piceae* complex are similar and difficult to distinguish in most cases. The sexual morph of *O. sugadairense* was very similar to that of *O. piceae* and *O. breviusculum* (Table 3.2). However, the asexual morph of the present fungus had unique morphological characteristics, such as its color (Table 3.1) and the shape of its synnemata (Table 3.4). The middle of the synnema stipe was relatively narrow, which is reflected in the ratio of the synnema base width to middle width (Table 3.4). This characteristic has not yet been reported in other members of the *O. piceae* complex and we were able to use it to distinguish the present fungus from *O. piceae* and *O. breviusculum*. Our results supported previous studies that synnematous

features are important criteria for *O. piceae* complex species recognition (Olchowecki and Reid 1974; Upadhyay and Kendrick 1975; Halmschlager et al. 1994; Harrington et al. 2001; Ohtaka et al. 2002a; Linnakoski et al. 2010); that is, the synnemata are composed of a robust stipe in *O. subalpinum*, while they are short and hyaline in *O. rachisporum* (Table 3.1).

Ophiostoma sugadairense was heterothallic and had two mating types, "+" and "–". The results of mating compatibility tests with mating type "A" and "B" isolates of *O. breviusculum* and *O. piceae* revealed that the two mating types, "+" and "–", of the present fungus could be considered mating types "A" and "B", respectively (Table 3.5). Based on results of the mating compatibility tests, the present fungus was considered to be reproductively isolated from *O. piceae*, *O. breviusculum*, and *O. subalpinum.* The present fungus sometimes mated with these species, but we considered these to be instances of interspecific mating for the following reasons. First, *O. sugadairense* required more time (about 2 mo) to produce perithecia when mated with the other three species than when mated with isolates of *O. sugadairense* (2 wk). Second, single ascospore F1 progeny isolates obtained from perithecia originating from two crosses between *O. sugadairense* (YCC-785; "A" mating type) and *O. piceae* (AU135-1; "B" mating type) exhibited abnormal colony characteristics, which were not found in either species (Fig. 3.2). Third, single ascospore F1 progeny isolates obtained from the cross between *O. sugadairense* (YCC-785) and *O. piceae* (AU135-1) did not produce perithecia after mating with each other. The results of the mating compatibility tests in the present study were similar to those in previous studies showing partial interfertility among *O. ulmi*, *O. novo-ulmi*, and *O. himal-ulmi* (Brasier and Mehrotra 1995; Brasier et al. 1998), between *O. piceae* and *O. canum* (Harrington et al. 2001), and between *O. piceae* and *O. breviusculum* (Chung et al. 2006).

In genetic mapping of mating type loci with amplified fragment length polymorphism markers and sequence analysis of mating type loci of *O. novo-ulmi* and *O. ulmi*, Paoletti et al. (2006) suggested that horizontal transfer of the MAT-1 locus frequently occurred from *O. ulmi* to *O. novo-ulmi*. Duong et al. (2016) developed and applied mating type markers to determine the mating type of isolates of *Leptographium* sensu lato. Wilken et al. (2012) also developed mating type markers to evaluate the distribution of the sexual compatibility types of *O. quercus* in different geographical areas. Therefore, future sequencing and characterization of mating type loci of *O. sugadairense* should be performed to clarify the evolution of the reproduction of this fungus.

No production of perithecia was expected after mating between the same mating type types of *O. sugadairense* and *O. piceae*. However, a few perithecia with ascospore production were observed in one cross between *O. sugadairense* (YCC-785; "A" mating type) and *O. piceae* (H2181; "A" mating type) (Table 3.5). This phenomenon, of a few perithecia being produced between the same types of isolate, was also reported in some isolates of *O. quercus* (e.g., CMW 2521, CMW 17258) (Wilken et al. 2012). Wilken et al. (2012) demonstrated that fragments of both idiomorphs were found in isolates of the opposite mating type of *O. quercus*. The MAT B isolate CMW 2521 had a large fragment of the *MAT1-1-3* (766 bp) and *MAT1-1-1* (712 bp) genes, in addition to the *MAT1-2-1* gene. They considered that the presence of two almost complete copies of the *MAT1-1* specific genes explains the ability of the isolate to act as both a MAT A and a MAT B strain. Therefore, the same or similar phenomena may occur between *O. sugadairense* and *O. piceae*.

In this study, molecular analysis revealed that *O. piceae* is polyphyletic. In the β-tubulin gene phylogeny and phylogeny of the concatenated ITS nrDNA, β-tubulin,

and EF-1α genes, four Japanese isolates of *O. piceae*, YCC-563, YCC-701, YCC-718, and YCC-731, grouped with the clade containing the ex-type isolate of *O. piceae* (CBS 108.21), excluding isolate YCC-637. However, they were located in separate groups in the EF-1α phylogeny. The five isolates YCC-563, YCC-637, YCC-701, YCC-718, and YCC-731, obtained from Koyama's spruce, Jack pine, eastern white pine, and Japanese white pine, were collected between 2005 and 2008 in Sugadaira, Nagano Prefecture, Japan. Morphological characteristics of Japanese isolates of *O. piceae* used in this study fit the description of *O. piceae* by Hunt (1956) and Upadhyay (1981). However, isolates of *O. piceae* from Yezo spruce (YCC-69, YCC-120) and Japanese larch (YCC-299, YCC-301) have a larger perithecial base than previously described (Yamaoka et al. 1997, 1998). Therefore, further investigation is required to clarify the taxonomic position of Japanese *O. piceae* isolates from conifer sources.

Most species of the *O. piceae* complex isolated from conifers are associated with bark beetles, although some caused sapwood staining without bark beetle attack (Mathiesen-Käärik 1960; Griffin 1968). For example, *O. canum* is known to be specifically associated with *Tomicus minor* that attacks *Pinus* spp. (Mathiesen 1950; Rennerfelt 1950; Mathiesen-Käärik 1953; Masuya et al. 1999); *O. subalpinum* consistently appears to be associated with *Cryphalus* spp. infested *Abies* spp. (Ohtaka et al. 2002a, b; Yamaoka et al. 2004); and *O. breviusculum* was isolated from *Ips subelongatus* and *Dryocoetes baikalicus*, which infested *L. kaempferi* (Chung et al. 2006). The present fungus was mainly isolated from *Polygraphus kisoensis* infesting *L. kaempferi*, although isolates were obtained from other bark beetles (as was *Ophiostoma* cf. *piceae* in Tokumasu 2009). Due to the lack of extensive isolation studies, it remains unclear whether this new species has a specific relationship with *P.*

kisoensis. However, *O. sugadairense* appeared to be strongly associated with *P. kisoensis*. Further studies are required to clarify the association of this species with bark beetles.

Chapter 4. Morphological study and mating compatibility tests of Group D

Phylogenetic analyses on the ITS nrDNA region, the partial β-tubulin, EF-1α genes and concatenated ITS nrDNA, β-tubulin, EF-1α genes conducted in the Chapter 2 showed that Group D was monophyletic and distinguishable from other *O. piceae* complex species. In this chapter, morphological studies and mating compatibility tests of Group D were conducted to determine the taxonomic status of this group.

4-1. Materials and Methods

4-2. Results

4-3. Taxonomy

4-4. Discussion

Contents of this chapter are not disclosed yet.

Chapter 5. Morphological study and mating compatibility tests of *Ophiostoma breviusculum*, Group A and Group B

Phylogenetic analyses on the ITS nrDNA region, the partial β-tubulin, EF-1α genes and concatenated ITS nrDNA, β-tubulin, EF-1α genes conducted in the Chapter 2 showed that *O. breviusculum*, Group A and Group B were monophyletic and distinguishable from other *O. piceae* complex species. In this chapter, comprehensive morphological study and mating compatibility tests among *O. breviusculum*, Group A and Group B were conducted to determine their taxonomic status.

5-1. Materials and Methods

5-2. Results

5-3. Discussion

Contents of this chapter are not disclosed yet.

Chapter 6. General discussion

6-1. Concept for species recognition

6-2. Species delineation in the *Ophiostoma piceae* complex based on molecular and morphological evidence

6-3. Contribution of taxonomic study on forest pathology

Contents of this chapter are not disclosed yet.

Summary

Contents of summary are not disclosed yet.

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References

Aoshima K, 1965. *Studies on wood staining fungi of Japan* (In Japanese with English summary). PhD thesis, University of Tokyo, Tokyo, Japan.

Aoshima K, Hayashi Y, 1956. A blue stain fungus of *Picea jezoensis*, *Ophiostoma bicolor* Davidson et Wells. *Annals of the Phytopathological Society of Japan* 21: 43.

Avise JC, Wollenberg K, 1997. Phylogenetics and the origin of species. *Proceedings of the National Academy of Sciences of the United States of America* 94: 7748–7755.

Baldauf SL, Roger AJ, Wenk-Siefert I, Doolittle WF, 2000. A kingdom-level phylogeny of eukaryotes based on combined protein data. *Science* 290: 972–977; http://dx.doi.org/10.1126/science.290.5493.972.

Bao D, Kinugasa S, Kitamoto Y, 2004. The biological species of oyster mushrooms (*Pleurotus* spp.) from Asia based on mating compatibility test. *Journal of Wood Science* 50: 162–168; http://dx.doi.org/10.1007/s10086-003-0540-z.

Bermer K, Wanntorp HE, 1979. Geographic populations or biological species in phylogeny reconstruction? *Systematic Zoology* 28: 220–224.

Brasier CM, 1993. The genetic system as a taxonomic tool: gene flow, molecular variation and sibling species in the *Ophiostoma piceae-Ophiostoma ulmi* complex and its taxonomic and ecological significance. In: Wingfield MJ, Seifert KA, Webber J (eds) Ceratocystis *and* Ophiostoma*: taxonomy ecology and pathogenicity.* American Phytopathological Society, Minnesota, pp 77–92.

Brasier CM, Gibbs JN, 1975. Highly fertile form of the aggressive strain of *Ceratocystis ulmi*. *Nature* 257: 128–131.

Brasier CM, Kirk SA, 1993. Sibling species within *Ophiostoma piceae*. *Mycological*

Research 97: 811–816; http://dx.doi.org/10.1016/S0953-7562(09)81156-8.

Brasier CM, Kirk SA, Pipe N, Buck KW, 1998. Rare interspecific hybrids in natural populations of the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi*. *Mycological Research* 102: 45–57; http://dx.doi.org/10.1017/S0953756297004541.

Brasier CM, Mehrotra MD, 1995. *Ophiostoma himal-ulmi* sp. nov., a new species of Dutch elm disease fungus endemic to the Himalayas. *Mycological Research* 99: 205–215; http://dx.doi.org/10.1016/S0953-7562(09)80887-3.

Brasier CM, Stephens TM, 1993. Temperature-growth responses distinguish the OPC and OPH sibling species within '*Ophiostoma piceae*'. *Mycological Research* 87: 1416–1418; http://dx.doi.org/10.1016/S0953-7562(09)80209-8.

Butin H, 1996. Krankheiten der Wald-und Parkbäume. *Diagnose Biologie Bekämpfung*. 3. *Auflage.* Georg Thieme Verlag, Stuttgart, Deutschland, New York, USA, pp 261.

Carlier F, Decock C, Jacobs K, Maraite H, 2006. *Ophiostoma arduennense* sp. nov. (Ophiostomatales, Ascomycota) from *Fagus sylvatica* in southern Belgium. *Mycological Research* 110: 801–810; http://dx.doi.org/10.1016/j.mycres.2006.03.010.

Chaverri P, Castlebury LA, Samuels GJ, Geiser DM, 2003. Multilocus phylogenetic structure within the *Trichoderma harzianum Hypocrea lixii* complex. *Molecular Phylogenetics and Evolution* 27: 302–313; http://dx.doi.org/10.1016/S1055-7903(02)00400-1

Chung WH, Kim J, Yamaoka Y, Uzunovic A, Masuya H, Breuil C, 2006. *Ophiostoma breviusculum* sp. nov. (Ophiostomatales, Ascomycota) is a new species in the *Ophiostoma piceae* complex associated with bark beetles infesting larch in Japan.

Mycologia 98: 801–814; http://dx.doi.org/10.3852/mycologia.98.5.801.

Crooke M, Bevan D, 1957. Note on the first British occurrence of *Ips cembrae* Heer (Col., Scolytidae). *Forestry* 30: 21–28.

Daniel HM, Meyer W, 2003. Evaluation of ribosomal RNA and actin gene sequences for the identification of ascomycetous yeasts. *International Journal of Food Microbiology* 86: 61–78; http://dx.doi.org/10.1016/S0168-1605(03)00248-4 .

De Queiroz K, 1985. The ontogenetic method for determining character polarity and its relevance to phylogenetic systematic. *Systematic zoology* 34: 280–299.

De Queiroz K, 1998. The general lineage concept of species, species criteria, and the process of speciation: A conceptual unification and terminological recommendations. In: Howard DJ and Berlocher SH (eds), *Species and speciation*. Oxford University Press, New York, pp 57–75.

Dettman JR, Jacobson DJ, Taylor JW, 2003. A multilocus genealogical approach to phylogenetic species recognition in the model eukaryote *Neurospora*. *Evolution international journal of organic evolution* 57: 2703–2720; http://dx.doi.org/10.1554/03-073.

De Beer ZW, Wingfield MJ, 2013. Emerging lineages in the Ophiostomatales. In: Seifert KA, De Beer ZW, Wingfield MJ (eds), *The Ophiostomatoid Fungi: expanding frontiers*. *CBS Biodiversity Series* 12. CBS-KNAW Fungal Biodiversity Centre, Utrecht, pp 21–46.

De Beer ZW, Wingfield BD, Wingfield MJ, 2003b. The *Ophiostoma piceae* complex in the Southern Hemisphere: a phylogenetic study. *Mycological Research* 107: 469–476; http://dx.doi.org/10.1017/S0953756203007445.

Donoghue MJ, 1985. A critique of the biological species concept and recommendations for a phylogenetic alternative. *Bryologist* 88: 172–181.

Dowding, 1969. The dispersal and survival of spores of fungi causing blue-stain in pine. *Transactions of the British Mycological Society* 52: 125–137.

Duong TA, De Beer ZW, Wingfield BD, Wingfield MJ, 2016. Mating type markers reveal high levels of heterothallism in *Leptographium sensu lato*. *Fungal Biology* 120: 538–546; http://dx.doi.org/10.1016/j.funbio.2016.01.001.

Francke-Grosmann H, 1967. Ectosymbiosis in wood-inhabiting insects. In Henry SM (ed), *Symbiosis*. Academic Press, New York, pp 141–205.

Fujimoto T, Kita K, Uchiyama K, Kuromaru M, Akutsu H, Oda K, 2006b. Age trends in the genetic parameters of wood density and the relationship with growth rates in hybrid larch (*Larix gmelinii* var. *japonica* and *L. kaempferi*) F1. *Journal Forestry Research* 11: 157–163; http://dx.doi.org/ 10.1007/s10310-005-0200-9.

Furniss M, Solheim H, Christiansen E, 1990. Transmission of blue-stain fungi by *Ips typographus* (Coleoptera: Scolytidae) in Norway spruce. *Annals of the Entomological Society of America* 83: 712–716.

Gardes M, Bruns TD, 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2: 113–118; http://dx.doi.org/10.1111/j.1365-294x.1993.tb00005.x.

Geiser DM, Frisvad JC, Taylor JW, 1998. Evolutionary relationships in *Aspergillus* section *Funigati* inferred from partial β-tubulin and hydrophobin DNA sequences. *Mycologia* 90: 831– 845; http://dx.doi.org/10.2307/3761325.

Georgévitch P, 1926. *Ceratostomella quercus* n. sp. *Comptes rendus hebdomadaires des séances de l'Académie des Sciences* 183: 759–761.

Gibbs JN, 1993. The biology of ophiostomatoid fungi causing sapstain in trees and freshly cut logs. In: Wingfield MJ, Seifert KA, Webber J (eds), Ceratocystis *and*

Ophiostoma*: taxonomy ecology and pathogenicity*. American Phytopathological Society, Minnesota, pp 141–151.

Gorton C, Kim SH, Henricot B, Webber J, Breuil C, 2004. Phylogenetic analysis of the bluestain fungus *Ophiostoma minus* based on partial ITS rDNA and β-tubulin gene sequences. *Mycological Research* 108: 759–765; http://dx.doi.org/10.1017/S0953756204000012.

Griffin HD, 1968. The genus *Ceratocystis* in Ontario. *Canadian Journal of Botany* 46: 689–718; http://dx.doi.org/10.1139/b68-094.

Grobbelaar JW, Aghayeva D, De Beer ZW, Bloomer P, Wingfield MJ, Wingfield BD, 2009. Delimitation of *Ophiostoma quercus* and its synonyms using multiple gene phylogenies. *Mycological Progress* 8: 221−236; http://dx.doi.org/10.1007/s11557-009-0594-4.

Grobbelaar JW, De Beer ZW, Bloomer P, Wingfield MJ, Wingfield BD, 2010. *Ophiostoma tsotsi* sp. nov., a wound-infesting fungus of hardwood trees in Africa. *Mycopathologia* 169: 413–423; http://dx.doi.org/10.1007/s11046-009-9267-8.

Hafez M, 2012. *Exploring the rns gene landscape in ophiostomatoid fungi and related taxa: Molecular characterization of mobile genetic elements and biochemical characterization of intron-encoded homing endonucleases.* PhD thesis, University of Manitoba, Manitoba, Canada.

Halmschlager E, Messner R, Kowalski T, Prillinger H, 1994. Differentiation of *Ophiostoma piceae* and *Ophiostoma quercus* by morphology and RAPD analysis. *Systematic and Applied Microbiology* 17: 554–562; http://dx.doi.org/10.1016/S0723-2020(11)80076-7.

Hamaya T, Kurahashi A, 1981. Breeding of larch by species hybridization in Japan. In: Krugman SL, Katsutapp M (eds) In: Proceedings of the 17th IUFRO

World congress, Kyoto, 6-7 September, pp 157–168.

Harrington TC, McNew DL, 1997. Self-fertility and unidirectional mating type switching in *Ceratocystis coerulescens*, a filamentous ascomycete. *Current Genetics* 32: 52–59; http://dx.doi.org/10.1007/s002940050247.

Harrington TC, McNew D, Steimel J, Hofstra D, Farrell R, 2001. Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia* 93: 111–136; http://dx.doi.org/10.2307/3761610.

Held BW, Thwaites JM, Farrell RL, Blanchette RA, 2003. Albino strains of *Ophiostoma* species for biological control of sapstaining fungi. *Holzforschung* 57: 237–242; https://doi.org/10.1515/HF.2003.036.

Helgason T, Watson IJ, Young JPW, 2003. Phylogeny of the *Glomerales* and *Diversisporales* (Fungi: Glomeromycota) from actin and elongation factor 1-alpha sequences. *FEMS Microbiology Letters* 229: 127–132; https://doi.org/10.1016/S0378-1097(03)00802-4.

Hibbett DS, Nakai YF, Tsuneda A, Donoghue MJ, 1995. Phylogenetic diversity in shiitake inferred from nuclear ribosomal DNA sequences. *Mycologia* 87: 618–638; https://doi.org/10.2307/3760806.

Huelsenbeck JP, Bull JJ, Cunningham CW, 1996. Combining data in phylogenetic analysis. *Trends in Ecology and Evolution* 11: 152−158; http://dx.doi.org/10.1016/0169-5347(96)10006-9.

Hunt J, 1956. Taxonomy of the genus *Ceratocystis*. *Lloydia* 19: 1–59.

Hyun MW, Kim SH, Suh DY, Kwon HW, Kim JY, 2012. Beta-tubulin gene based on molecular marker for wood-Staining.

Isebrands JG, Hunt CM, 1975. Growth and wood properties of rapid–grown Japanese larch. *Wood and Fiber* 7: 119–128.

Ito K, 1973. Diseases caused by *Ceratocystis*. In: Ito K (ed), *Pathology of forest trees II* (in Japanese). Norin Shuppan, Tokyo, pp 26–44.

Jacobs K, Bergdahl DR, Wingfield MJ, Halik S, Seifert KA, Bright DE, Wingfield BD, 2004. *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* 108: 411–418; http://dx.doi.org/10.1017/S0953756204009748.

Jacobs K, Kirisits T, 2003. *Ophiostoma kryptum* sp. nov. from *Larix deciduas* and *Picea abies* in Europe, similar to *O. minus*. *Mycological Research* 107: 1231–1242; http://dx.doi.org/10.1017/S0953756203008402.

Jacobs K, Wingfield MJ, 2001. *Leptographium* species: Tree pathogens, insect associates, and agents of blue-stain. American Phytopathological Society, Minnesota, pp 207.

Jankowiak R, Bilański P, 2013b. Ophiostomatoid fungi associated with root–feeding bark beetles on Scots pine in Poland. *Forest Pathology* 43: 422–428; http://dx.doi.org/10.1111/efp.12049.

Jankowiak R, Kolařík M, 2011. Ophiostomatoid fungi isolated from fallen shoots of Scots pine pruned by *Tomicus* species in Poland. *Acta Mycologica* 46: 201–210; http://dx.doi.org/10.5586/am.2011.013.

Kamgan Nkuekam G, De Beer ZW, Wingfield MJ, Mohammed C, Carnegie AJ, Pegg GS, Roux J, 2011. *Ophiostoma* species (Ophiostomatales, Ascomycota), including two new taxa on eucalypts in Australia. *Australian Journal of Botany* 59: 283–297; http://dx.doi.org/10.1071/BT10231.

Kamgan Nkuekam G, De Beer ZW, Wingfield MJ, Roux J, 2012. A diverse assemblage of *Ophiostoma* species, including two new taxa on eucalypt trees in South Africa. *Mycological Progress* 11: 515–533;

http://dx.doi.org/10.1007/s11557-011-0767-9.

Kamgan Nkuekam G, Jacobs K, De Beer ZW, Wingfield MJ, Roux J, 2008. *Pesotum australi* sp. nov. and *Ophiostoma quercus* associated with *Acacia mearnsii* trees in Australia and Uganda. *Australasian Plant Pathology* 37: 406–416; http://dx.doi.org/10.1071/AP08027.

Kamgan Nkuekam G, Solheim H, De Beer ZW, Grobbelaar JW, Jacobs K, Wingfield MJ, Roux J, 2010. *Ophiostoma* species, including *Ophiostoma borealis* sp. nov., infecting wounds of native broad–leaved trees in Norway. *Cryptogamie Mycologie* 31: 285–303.

Katoh K, Standly DM, 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* 30: 772–780; http://dx.doi.org/10.1093/molbev/mst010.

Kim JJ, Kim SH, Lee S, Breuil C, 2003. Distinguishing *Ophiostoma ips* and *Ophiostoma montium*, two bark beetle-associated fungi. *FEMS Microbiology Letters* 222: 187–192; http://dx.doi.org/10.1016/S0378-1097(03)00304-5.

Kim GH, Kim JJ, Lim YW, Breuil C, 2005. Ophiostomatoid fungi isolated from *Pinus radiate* logs imported from New Zealand to Korea. *Canadian Journal of Botany* 83: 272–278; http://dx.doi.org/10.1139/b04-170.

Kim SH, Uzunovic A, Brueil C, 1999. Rapid detection of *Ophiostoma piceae* and *O. quercus* in stained wood by PCR. *Applied and Environmental Microbiology* 65: 287–290.

Kirisits T, 2004. Fungal associates of European bark beetles with special emphasis on the ophiostomatoid fungi. In: Lieutier F, Day KR, Battisti A, Grégoire JC, Evans H (eds), *Bark and Wood Boring Insects in Living Trees in Europe*: *a synthesis*, Kluwer Academic, Dordrecht, Netherlands, pp 185–235.

Lanave C, Preparata G, Saccone C, Serio G, 1984. A new method for calculating evolutionary substitution rates. *Journal of Molecular Evolution* 20: 86–93; http://dx.doi.org/10.1007/bf02101990.

Lee S, Kim JJ, Fung S, Breuil C, 2003. A PCR-RFLP marker distinguishing *Ophiostoma clavigerum* from morphologically similar *Leptographium* species associated with bark beetles. *Canadian Journey of Botany* 81: 1104–1112; http://dx.doi.org/10.1139/b03-101.

Levin D, 1978. The nature of plant species. *Science* 204: 381–384.

Levin D, 1978. The origin of isolating mechanisms in flowering plants. *Evolutionary Biological* 11: 185–317.

Lieutier F, 2002. Mechanisms of resistance in conifers and bark beetle attack strategies. In: Wagner MR, Clancy KM, Lieutier F, Paine TD (eds.), *Mechanisms and Deployment of Resistance in Trees to Insects*, Kluwer Academic Publishers, Dordrecht, pp 31–77.

Linnakoski R, De Beer ZW, Ahtiainen J, Sidorov I, Niemela P, Pappinen A, Wingfield MJ, 2010. *Ophiostoma* spp. associated with pine and spruce infesting bark beetles in Finland and Russia. *Persoonia* 25: 72–93; http://dx.doi.org/10.3767/003158510X550845.

Linnakoski R, De Beer ZW, Duong TA, Niemelä P, Pappinen A, Wingfield MJ, 2012. *Grosmannia* and *Leptographium* spp. associated with conifer–infesting bark beetles in Finland and Russia, including *L. taigense* sp. nov. *Antonie van Leeuwenhoek* 102: 375–399; http://dx.doi.org/10.1007/s10482-012-9747-6.

Linnakoski R, De Beer ZW, Rousi M, Niemela P, Pappinen A, Wingfield MJ, 2008. Fungi including *Ophiostoma karelicum* sp. nov., associated with *Scolytus ratzeburgi* infesting birch in Finland and Russia. *Mycological Research* 112: 1475–

1488; http://dx.doi.org/10.1016/j.mycres.2008.06.007.

Linnakoski R, De Beer ZW, Rousi M, Solheim H, Wingfield MJ, 2009. *Ophiostoma denticiliatum* sp. nov. and other *Ophiostoma* species associated with the birch bark beetle in southern Norway. *Persoonia* 23: 9–15; http://dx.doi.org/10.3767/003158509X468038.

Masuya H, Kaneko S, Yamaoka Y, Ohsawa M, 1999. Comparisons of ophiostomatoid fungi associated with *Tomicus piniperda* and *T. minor* in Japan red pine. *Journal of Forestry Research* 4: 131–135; http://dx.doi.org/10.1007/bf02762237.

Massoumi Alamouti S, Kim J, Humble L, Uzunovic A, Breuil C, 2007. Ophiostomatoid fungi associated with the northern spruce engraver, *Ips perturbatus*, in western Canada. *Antonie van Leeuwenhoek* 91: 19–34; http://dx.doi.org/10.1007/s10482-006-9092-8.

Massoumi Alamouti S, Tsui CKM, Breuil C, 2009. Multigene phylogeny of filamentous ambrosia fungi associated with ambrosia and bark beetles. *Mycological Research* 113: 822–835; http://dx.doi.org/10.1016/j.mycres.2009.03.003.

Mathiesen A, 1950. Über einige mit Borkenkäfern assoziierte Bläuepilze in Schweden. *Oikos* 2: 275–308; http://dx.doi.org/10.2307/3564798.

Mathiesen A, 1951. Einige neue *Ophiostoma*-arten in Schweden. *Svensk Botanisk Tidskrift* 45: 203–232.

Mathiesen-Käärik A, 1953. Eine Übersicht über die gewöhnlichsten mit Borkenkäfern assoziierten Bläuepilze in Schweden und einige für Schweden neue Bläuepilze. *Medde landen från Statens Skogforskningsinstitutut* 43: 1–74.

Mathiesen-Käärik A, 1960. Studies on the ecology, taxonomy and physiology of Swedish insect-associated blue stain fungi, especially the genus *Ceratocystis*.

Oikos 11: 1–25; http://dx.doi.org/10.2307/3564881.

Mayden RL, 1997. A hierarchy of species concepts: The denouement in the saga of the species problem. In: Claridge MF, Dawah HA, Wilson MR (eds), *Species: The Units of Biodiversity*, Chapman and Hall, London, pp 381–424.

Mayr E, 1957. Difficulties and importance of the biological species concept. In Mayr E. (ed), *The species problem*. American Association for the Advancement of Science, Washington: A.A.A.S., pp 371–388.

Morelet M, 1992. *Ophiostoma querci* sur chêne en France. *Annales de la Societe des Sciences Naturelles et d'Archeologie de Toulon et du Var* 44: 109–112.

Münch E, 1907. Die Blaufäule des Nadelholzes. I-II. *Naturwissenschaftliche Zeitschrift für Land- und Forstwirtschaft* 5: 531–573.

Nagamitsu T, Nagasaka K, Yoshimaru H, Tsumura Y, 2014a. Provenenance tests for survival and growth of 50-year-old Japanese larch (*Larix kaempferi*) trees related to climatic conditions in Japan. *Tree Genetics and Genomes*10: 87–99; http://dx.doi.org/10.1007/s11295-013-0666-0.

Nisikado Y, Yamauti K, 1935. Contributions to the knowledge of the sap stains of wood in Japan. III. Studies on *Ceratostomella piceae* Münch, the cause of a blue stain of pine trees. *Berichte des Ohara Instituts für Landwirtschaftliche Forschungen* 6: 539–560.

Nylander JAA, 2004. *MrModeltest 2.3.* Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala.

O' Donnell K, Cigelnik E, 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* 7: 103–116; http://dx.doi.org/10.1006/mpev.1996.0376.

O' Donnell K, Nirenberg HI, Aoki T, Cigelnik, E, 2000. A multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. *Mycoscience* 41: 61–78.

Ohtaka N, Masuya H, Kaneko S, Yamaoka Y, 2002a. Two *Ophiostoma* species associated with bark beetles in wave-regenerated *Abies veitchii* forests in Japan. *Mycoscience* 43: 151–157; http://dx.doi.org/10.1007/s102670200022.

Ohtaka N, Masuya H, Kaneko S, Ohsawa M, 2002b. Ophiostomatoid fungi associated with bark beetles on *Abies veitchii* in wave-regenerated forests. *The Journal of Forestry Research* 7: 145–151; http://dx.doi.org/10.1007/bf02762603.

Ohtaka N, Masuya H, Yamaoka Y, Kaneko S, 2006. Two new *Ophiostoma* species lacking conidial states isolated from bark beetles and bark beetle-infested *Abies* species in Japan. *Canadian Journal of Botany* 84: 282–192; http://dx.doi.org/10.1139/b05-164.

Ohtani Y, 1988. Ophiostomatales Benny et Kimbrough. In: Otani Y (ed), *Mycological Flora of Japan* (in Japanese). Yokendo Press, Tokyo, pp 134–147.

Olchowecki A, Reid J, 1974. Taxonomy of the genus *Ceratocystis* in Manitoba. *Canadian Journal of Botany* 52: 1675–1711; http://dx.doi.org/10.1139/b74-222.

Paine TD, Raffa KF, Harrington TC, 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* 42: 179–206; http://dx.doi.org/10.1146/annurev.ento.42.1.179.

Paoletti M, Buck KW, Brasier CM, 2006. Selective acquisition of novel mating type and vegetative incompatibility genes via interspecies gene transfer in the globally invading eukaryote *Ophiostoma novo-ulmi*. *Molecular Ecology* 15: 249–262; http://dx.doi.org/10.1111/j.1365-294X.2005.02728.x.

Peng X, Kajimura H, Shibata E, 1996. Response of Japanese red pine to inoculation

with a blue stain fungus, *Ceratocystis piceae*. *Journal of Forest Research* 1: 41−44; http://dx.doi.org/10.1007/bf02348339.

Perkins DD, 1994. How should the infertility of interspecies crosses be designated? *Mycologia* 86: 758–761; http://dx.doi.org/10.2307/3760588.

Perry HJ, Cook DB, 1965. Pulpwood for 1995. *Pulp paper* 39: 33–34.

Pipe ND, Buck KW, Brasier CM, 1995. Genomic fingerprinting supports the separation of *Ophiostoma piceae* into two species. *Mycological Research* 99: 1182–1186; http://dx.doi.org/10.1016/S0953-7562(09)80274-8.

Przybyl K, Morelet M, 1993. Morphological differences between *Ophiostoma piceae* and *O. querci*, and among *O. querci* isolates. *Cryptogamie Mycologie* 14: 219–228.

Redfern DB, Stoakley JT, Steele H, Minter DW, 1987. Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae*. *Plant Pathology* 36: 467–480; http://dx.doi.org/10.1111/j.1365-3059.1987.tb02264.x.

Rennerfelt E, 1950. Über den Zusammenhang Zwischen dem Verblauen des Holzes und den Insekten. *Oikos* 2: 120–137.

Ronquist F, Huelsenbeck JP, 2003. MrBayes 3: *Bayesian phylogenetic inference under mixed models*. *Bioinformatics* 19: 1572–1574; http://dx.doi.org/10.1093/bioinformatics/btg180.

Rosen DE, 1978. Vicariant patterns and historical explanation in biogeography. *Systematic Zoology* 27: 159–188; https://doi.org/10.2307/2412970.

Rosen DE, 1979. Fishes from the uplands and intermontane basins of Guatemala: revisionary studies and comparative geography. *Bulletin of the American Museum of Natural History* 162: 267–376.

Schardl CL, Leuchtmann A, Tsai HF, Collett MA, Watt DM, Scott DB, 1994. Origin of a fungal symbiont of perennial ryegrass by interspecific hybridization of a

mutualist with the ryegrass choke pathogen, *Epichloë typhina*. *Genetics* 136: 1307–1317.

Schroeder S, Kim SH, Cheung WT, Sterflinger K, Breuil C, 2001. Phylogenetic relationship of *Ophiostoma piliferum* to other sapstain fungi based on the nuclear rRNA gene. *FEMS Microbiology Letters* 195: 163–167; http://dx.doi.org/10.1111/j.1574-6968.2001.tb10515.x.

Seifert KA, 1993. Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In: Wingfield MJ, Seifert KA and Webber JF (eds). *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*. American Phytopathological Society , Minnesota, pp 141–151.

Simpson GG, 1951. The species concept. *Evolution* 5: 285–298.

Simpson GG, 1961. Principles of Animal Taxonomy. Columbia University Press, New York.

Singh BN, 2012. Concepts of species and models of speciation. *Current Science* 103: 784–790.

Solheim H, Krokene P, 1998. Growth and virulence of mountain pine beetle associated blue-stain fungi, *Ophiostoma clavigerum* and *Ophiostoma montium*. *Canadian Journal of Botany* 76: 561–566; https://doi.org/10.1139/b98-020.

Stauffer C, Kirisits T, Nussbaumer C, Pavlin R, Wingfield MJ, 2001. Phylogenetic relationships between the European and Asian eight spined larch bark beetle populations (Coleoptera, Scolyltidae) inferred from DNA sequences and fungal associates. *European Journal of Entomology* 98: 99–105.

Swofford DL, 2003. *PAUP*. Phylogenetic analysis using parsimony (*and other methods). version 4*. Sinauer Associates, Sunderland, Massachusetts.

Takahashi N, Nishiguchi C, 1966. Studies on the resistance of forest trees to the

red backed vole, *Clethrionomys rufocanus bedfordiae* (Thomas) (in Japanese with English summary). Bulletin of Tokyo University Forests 62:173–188.

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC, 2000. Phylogenetic species recognition and species concepts in Fungi. *Fungal Genetics and Biology* 31: 21–31; https://doi.org/10.1006/fgbi.2000.1228.

Tavaré S, 1986. Some probabilistic and statistical problems on the analysis of DNA sequences. *Lectures on Mathematics in the Life Sciences* 17: 57–86.

Tochinai Y, Sakamoto M, 1934. Ezomatsu-zai no seihen ni tsuite (Studies on blue-stain of wood of *Picea jezoensis*) (in Japanese). *Hokkaido Ringyo Kaiho* 32: 334–342.

Tokumasu S, 2009. An intensive investigation on the species diversity of microfungi within a small place (in Japanese). *IFO Research Communications* 23: 73–97.

Upadhyay HP, 1981. *A monograph of* Ceratocystis *and* Ceratocystiopsis. University of Georgia Press, Athens.

Upadhyay HP, Kendrick WB, 1975. Prodromus for a revision of *Ceratocystis* (Microascales, Ascomycetes) and its conidial states. *Mycologia* 67: 798–805; http://dx.doi.org/10.2307/3758340.

Uzunovic A, Webber JF, 1998. Comparison of bluestain fungi grown in vitro and in freshly cut pine billets. *European Journal of Plant Pathology* 28: 323–334; http://dx.doi.org/10.1111/j.1439-0329.1998.tb01187.x.

Uzunovic A, Seifert KA, Kim SH, Breuil C, 2000. *Ophiostoma setosum*, a common sapwood staining fungus from western North America, a new species of the *Ophiostoma piceae* complex. *Mycological Research* 104: 486–494; http://dx.doi.org/10.1017/S0953756299001446.

Vilgalys R, Sun BL, 1994. Ancient and recent patterns of geographic speciation in

the oyster mushroom *Pleurotus* revealed by phylogenetic analysis of ribosomal DNA sequences. *Proceedings of the National Academy of Sciences of the United States of America* 91: 4599–4603.

Villarreal M, Rubio V, De Troya MT, Arenal F, 2005. A new *Ophiostoma* species isolated from *Pinus pinaster* in the Iberian Peninsula. *Mycotaxon* 92: 259–268.

White MJD. 1978. Modes of Speciation. WH Freeman: San Francisco, USA, pp 1–455.

White TJ, Bruns T, Lee SB, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand D, Sninsky JJ, White TJ (eds), *PCR protocols: a guide to methods and applications*. Academic Press, San Diego, pp 315–322.

Whitney HS, 1982. Relationships between bark beetles and symbiotic organisms. In: Mitton JB and KB Sturgeon (eds). *Bark beetles in North American conifers*. Austin: University of Texas, pp 183–211.

Wiley EO, 1981. Phylogenetics. The Theory and Practice of Phylogenetic Systematics. Wiley Interscience, New York, pp 439.

Wilken PM, Steenkamp ET, Hall TA, De Beer ZW, Wingfield MJ, Wingfield BD, 2012. Both mating types in the heterothallic fungus *Ophiostoma quercus* contain MAT1-1 and MAT1-2 genes. *Fungal Biology* 116: 427–437; http://dx.doi.org/10.1016/j.funbio.2012.01.002.

Wood SL, Bright DE, 1992. A catalogue of Scolytidae and Platypodidae (Coleoptera), Part 2: taxonomic index. Volumes A and B. *Great Basin Naturalist Memoirs* 13: 1–1553.

Wulf A, Kowalski T, 1994. Die Wachstumsgeschwindigkeit als Unterscheidungsmerkmal zwischen *Ophiostoma piceae* and *Ophiostoma querci*.

European Journal of Plant Pathology 24: 123–127.

Yamaguchi T, Sasaki K, Matsuzaki S, 1991. Inoculation tests of *Ceratocystis piceae* to Japanese larch Wilt of inoculated trees (In Japanese). *Transactions of the 39th Annual Meeting of the Hokkaido Branch of Japan Forestry Society* 39: 76–78.

Yamaoka Y, Chung WH, Masuya H, Hizai M, 2009. Constant association of ophiostomatoid fungi with the bark beetle *Ips subelongatus* invading Japanese larch logs. *Mycoscience* 50: 165–172; http://dx.doi.org/10.1007/s10267-008-0468-7.

Yamaoka Y, Masuya H, Ohtaka N, Goto H, Kaneko S, Kuroda Y, 2004. *Ophiostoma* species associated with bark beetles infesting three *Abies* species in Nikko, Japan. *Journal of Forest Research* 9: 67–74; http://dx.doi.org/10.1007/s10310-003-0056-9.

Yamaoka Y, Wingfield MJ, Ohsawa M, Kuroda Y, 1998. Ophiostomatoid fungi associated with *Ips cembrae* in Japan and their pathogenicity to Japanese larch. *Mycoscience* 39: 367–378; http://dx.doi.org/10.1007/BF02460897.

Yamaoka Y, Wingfield MJ, Takahashi I, Solheim H, 1997. Ophiostomatoid fungi associated with the spruce bark beetle *Ips typographus* f. *aponicus* in Japan. *Mycological Research* 101: 1215–1227; http://dx.doi.org/10.1017/S0953756297003924.

Yan Z, Sun J, Don O, Zhang Z, 2005. The red turpentine beetle, *Dendroctonus valens* LeConte (Scolytidae): an exotic invasive pest of pine in China. *Biodiversity and Conversation* 14 1735–1760; http://dx.doi.org/10.1007/s10531-004-0697-9.

Yang Z, 1994. Estimating the pattern of nucleotide substitution. *Journal of Molecular Evolution* 39: 105–111; http://dx.doi.org/10.1007/bf00178256.

Yin ML, Wingfield MJ, Zhou XD, De Beer ZW, 2016. Multigene phylogenies and

morphological characterization of five new *Ophiostoma* spp. associated with spruce–infesting bark beetles in China. *Fungal biology* 120: 454–470; http://dx.doi.org/10.1016/j.funbio.2015.12.004.

Zanzot JW, De Beer ZW, Eckhardt LG, Wingfield MJ, 2010. A new *Ophiostoma* species from loblolly pine roots in the southeastern United States. *Mycological Progress* 9: 447–457; http://dx.doi.org/10.1007/s11557-010-0657-6.

Zink RM, McKitrick MC, 1995. The debate over species concepts and its implications for ornithology. *The Auk* 112: 701–719.

Zhou XD, De Beer ZW, Cibrian D, Wingfield BD, Wingfield MJ, 2004a. Characterization of *Ophiostoma* species associated with pine bark beetles from Mexico, including *O. pulvinisporum* sp. nov. *Mycological Research* 108: 690–698; http://dx.doi.org/10.1017/s0953756204009918.

Zwickl DJ, 2006. *Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion*. PhD thesis, University of Texas at Austin, Texas.