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Review

Taxonomy and pathogenicity of ophiostomatoid fungi associated with bark beetles infesting conifers in Japan, with special reference to those related to subalpine conifers

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ABSTRACT

Ophiostomatoid fungi are ambiguous. They belong to the Ascomycota, and were formerly treated as one family and grouped in one genus, but molecular phylogenic studies have shown that they are divided into two orders and their generic classification must be altered. Our research group conducted taxonomic studies on ophiostomatoid fungi, their association with bark beetles, and their pathogenicity against host trees. This review summarizes recent changes in the taxonomic treatment of these fungi and their relationships with bark beetles associated with subalpine conifers in Japan. Furthermore, methods for evaluating their virulence against conifer trees are discussed.

1. Introduction

Sapwood of conifer trees and logs is frequently stained blue or bluish-gray due to attacks by some species of bark beetle. This is known as blue stain, and the causative fungi as blue-stain fungi. Blue stain is caused mainly by ascomycetous fungi, which were formerly classified as *Ceratocystis* or *Ophiostoma* (Seifert 1993), although the taxonomic treatment of these groups differs among mycologists and over time. For example, Hunt (1956) treated them as one genus, *Ceratocystis*. Upadhyay (1981) separated *Ceratocystiopsis* from *Ceratocystis* based on morphological characteristics, although *Ceratocystiopsis* did not include blue-stain fungi. *Ceratocystis* and *Ophiostoma* were treated separately by von Arx (1974), de Hoog and Scheffer (1984), and Harrington (1987) based on differences in the conidiogenesis of asexual morphs, components of the cell wall, and tolerance to cycloheximide. Later comprehensive molecular phylogenic studies (Hausner et al. 1993a,b; Spatafora and Blackwell 1994) revealed that *Ceratocystis*

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belongs to the Ceratocystidaceae, Microascales, while *Ophiostoma* and *Ceratocystiopsis* belong to the Ophiostomataceae, Ophiostomatales. These taxa are frequently isolated from the same substrates and studied together, because they have similar ecological, physiological, and morphological characteristics. Therefore, these fungi are frequently termed "ophiostomatoid fungi" irrespective of their taxonomic positions (Wingfield et al. 1993) and despite their marked phylogenetic differences.

Zipfel et al. (2006) recognized three well-supported, monophyletic lineages based on molecular phylogenetic studies of *Ophiostoma* s. l. using the nuclear large subunit (LSU) rDNA and β-tubulin genes (βT). They were treated as distinct genera; e.g., *Ceratocystiopsis*, *Grosmannia*, and *Ophiostoma*. De Beer and Wingfield (2013) conducted a comprehensive molecular phylogenetic analysis of the sequences of the LSU and internal transcribed spacer (ITS) region of rDNA, and revealed that Ophiostomatales comprised six genera and 18 species complexes. The six genera were *Ophiostoma* s. str., *Raffaelea* s. str., *Ceratocystiopsis*, *Fragosphaeria*, *Graphilbum*, and *Leptographium* s. l. *Ceratocystis* s. l., *Cornuvesica*, *Knoxdaviesia* (= *Gondwanamyces*), *Custingophora*, and *Graphium* s. str. *Sphaeronaemella* were included in the Microascales (De Beer et al. 2013a). As a result, De Beer et al. (2013b) classified 397 species of 12 genera in the Ophiostomatales and the Microascales.

De Beer et al. (2014) conducted a phylogenic analysis based on DNA-sequence data of three regions—the 60S ribosomal protein RPL10 (60S), the nuclear ribosomal DNA LSU, and mini-chromosome maintenance complex component 7 (MCM7)—of 79 species residing in the aggregate genus *Ceratocystis* s. l., and recognized seven major groups therein. These seven groups were treated as distinct genera; i.e., *Ceratocystis* s. str., *Chalaropsis*, *Endoconidiophora*, *Thielaviopsis*, *Ambrosiella*, *Davidsoniella*, and *Huntiella*. De Beer et al. (2016) also conducted a phylogenic analysis using DNA-sequence data of the LSU, ITS, βT, and calmodulin genes (CAL) of *Ophiostoma* and *Sporothrix* species, together with related species. The results revealed that *Sporothrix* was a well-supported monophyletic lineage comprising 51 taxa and was distinct from *Ophiostoma* s. str.

Several ophiostomatoid fungi are closely associated with bark beetles (Scolytidae, Coleoptera). These insects construct galleries in the inner bark of trees for the purposes of reproduction. Numerous studies of the interrelationship between ophiostomatoid fungi and bark beetles have been conducted, particularly in North America, Europe, and Japan. Some bark beetles have relationships with specific ophiostomatoid fungi (Whitney 1982; Six 2003; Kirisits 2004). In particular, bark beetles with mycangia (organs used for carrying symbiotic fungi), have relationships with specific fungi, including ophiostomatoid fungi. For example, the mountain pine beetle (*Dendroctonus ponderosae* Hopkins) and the Jeffrey pine beetle (*D. jeffreyi* Hopkins) has a relationship with *Grosmannia clavigera* (R.C. Rob. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf. (= *Europhium clavigerum* R.C. Rob. & R.W. Davidson) (Whitney and Farris 1970; Six and Paine 1997). Some bark beetles without mycangia might have associations with specific ophiostomatoid fungi; e.g., *Ophiostoma canum* (Münch) Syd. & P. Syd. with *Tomicus minor* (Hartig) (Masuya et al. 1999). Although the specificity of association might differ among the various combinations of ophiostomatoid fungi and bark beetles, ophiostomatoid fungi maintain a specific relationship with bark beetles and/or host plants.

Mass attacks by bark beetles can cause serious damage to conifer trees. However, ophiostomatoid fungi associated with bark beetles are considered to be at least in part responsible for the mortality of attacked trees (Whitney 1982; Harrington 1993a; Paine et al. 1997). Ophiostomatoid fungi associated with bark beetles might be virulent towards host trees irrespective of their ability to cause blue stain; e.g., *G. clavigera* [as *O. clavigerum* (Rob.-Jeffr. & R.W. Davidson) T.C. Harr.] associated with the mountain pine beetle (Yamaoka et al. 1995; Solheim and Krokene 1998), *Leptographium* species causing root disease of conifers (Harrington and Cobb 1988), and *Endoconidiophora polonica* (Siemaszko) Z.W. de Beer, T.A. Duong & M.J. Wingf. [= *Ceratocystis polonica* (Siemaszko) C. Moreau] associated with *Ips typographus* L. in Europe (Solheim 1988) and *I. typographus japonicus* Niijima in Japan (Yamaoka et al. 2000) (Fig. 1). Thus, it is important to clarify the diversity of the fungi, the relationship between the fungi and the beetles, and their virulence to host plants.

In Japan, Kasai (1917) first reported a blue-stain fungus, *O. piliferum* (Fr.) Syd. & P. Syd. Subsequently, several studies of blue-stain fungi or ophiostomatoid fungi have been reported. One of the most extensive studies of the association between ophiostomatoid fungi and bark beetles was conducted by Aoshima (1965). He clarified the associations of many species of ophiostomatoid fungi and bark beetles invading soft and hard wood in Japan and reported a large number of undescribed species. Beginning in the late 1990s, our research group focused on the specific relationships of ophiostomatoid fungi with bark beetles (e.g., Yamaoka et al. 1997; Ohtaka et al. 2002a; Masuya et al. 2009) and described multiple new species (e.g., Masuya et al. 2003; Yamaoka et al. 2004b). As mentioned above, recent molecular phylogenetic studies have altered the taxonomic treatment of ophiostomatoid fungi in Japan. Masuya et al. (2013) summarized the ophiostomatoid fungi reported in Japan, which included 55 species of *Ophiostoma* and related fungi and 11 species of *Ceratocystis*. The *Ophiostoma* and related fungi were subdivided into seven groups based on their morphology and phylogeny. Two of these groups are now recognized as distinct genera, *Grosmannia* and *Ceratocystiopsis*. Further taxonomic revisions were subsequently conducted.

This paper reports additional information regarding the fungi associated with bark beetles infesting subalpine conifers in Japan. Moreover, several studies of the pathogenicity of ophiostomatoid fungi are described because of their importance in forest pathology.

2. Ophiostomatoid fungi associated with bark beetles infesting subalpine conifers in Japan

2.1. *Ophiostomatoid fungi from firs (Abies spp.)*

Ophiostomatoid fungi associated with bark beetles infesting *Abies* spp. trees in Japan were investigated by Aoshima (1965), Ohtaka et al. (2002a,b), and Yamaoka et al. (2004a,b). Aoshima

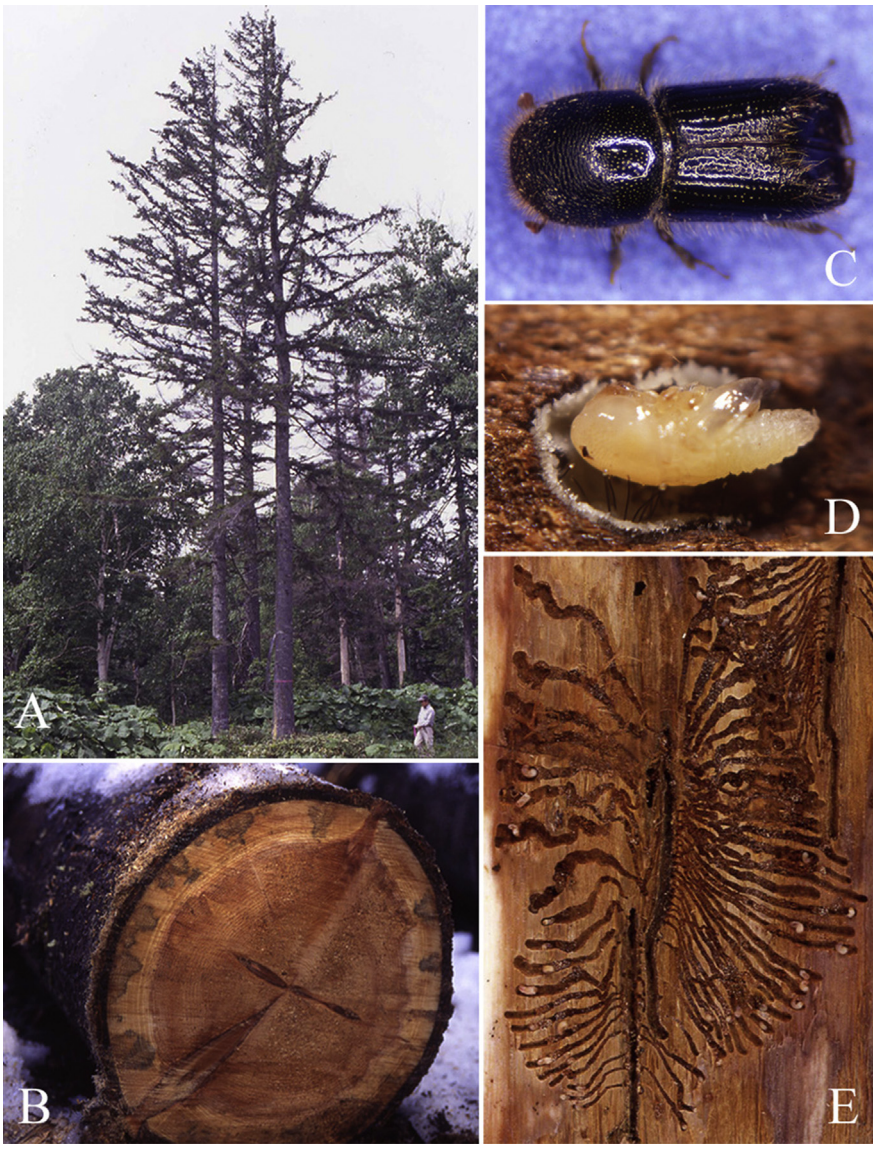

Fig. 1 – Yezo spruce infested by *Ips typographus japonicus*. A: Dying Yezo spruce trees infested by *I. typographus japonicus*. B: Blue staining of sapwood of Yezo spruce trees. C: An adult beetle of *I. typographus japonicus* (Courtesy Ms. N. Kimpara). D: Pupa in a pupal chamber lined with ophiostomatoid fungi [Adapted from Yamaoka (2002)]. E: Galleries of *I. typographus japonicus* in the inner bark of Yezo spruce.

(1965) surveyed mainly ophiostomatoid fungi associated with a bark beetle, *Polygraphus proximus* Blandford infesting *Abies sachalinensis* (F. Schmidt) Mast. in Hokkaido, northern Japan, while Ohtaka et al. (2002a, b) and Yamaoka et al. (2004a, b) examined fungi associated with bark beetles infesting *Abies mariesii* Mast., *A. veitchii* Lidl., and *A. homolepis* Siebold et Zucc. in central Honshu. Most of the revised information was presented by Masuya et al. (2013), but additional information regarding the fungi associated with bark beetles infesting *A. mariesii*, *A. veitchii*, and *A. homolepis* in Nikko, Tochigi Pref., central Honshu, Japan (Yamaoka et al. 2004a) is shown in Table 1.

Most of the species were unique and were reported only from bark beetles infesting *Abies* spp. in Japan; e.g., *Grosmannia abieticola* (Yamaoka & Masuya) Masuya & Yamaoka, *G. aoshimae* (Ohtaka, Masuya & Yamaoka) Masuya & Yamaoka, *Graphilbum microcarpum* (Yamaoka & Masuya) Z.W. de Beer & M.J. Wingf., *Gra. rectangulosporium* (Ohtaka, Masuya & Yamaoka), Z.W. de Beer & M.J. Wingf., *Ophiostoma nikkoense* Yamaoka & Masuya, and *O. subalpinum* Ohtaka & Masuya. Masuya et al. (2013) suggested that this is because *Abies* species are indigenous to Japan. The fungi previously identified as *Grosmannia europhioides* (E.F. Wright & Cain) Zipfel, Z.W. de Beer & M.J. Wingf. and its related species (*Ophiostoma* sp. D and *Ophiostoma* sp. P) reported in Yamaoka et al. (2004a) are now recognized as distinct species, *Grosmannia* spp. J-5, J-6, and J-7, respectively, according to Ando et al. (2016). *Grosmannia* spp. J-6 and J-7 were also reported only on *Abies* spp.

Because more than two species of bark beetle can be present on a single log, cross-contamination of fungi is possible. The major vectors of the fungi are shown in Table 1. As reported previously, *G. abieticola*, *G. davidsonii* (Olchow. & J. Reid)

Table 1 – Frequency of occurrence of ophiostomatoid fungi from bark beetles infesting firs (*Abies* spp.).

Species	*Cryphalus montanus*					*Polygraphus proximus*				*Dryocoetes hectographus*			*D. autographus*			*D. striatus*
	Am3[a]	Am4	Am5	Am6	Am8	Am1	Am2	Ah1	Ah2	Am1	Am2	Am3	Am6	Am7	Av1	Ah1
Grosmannia abieticola (*Ophiostoma* sp. J)[c]	1[b]	1		1	1	1	2			2	3	3	4	4	4	
G. aoshimae (*Ophiostoma* sp. B)	1	1			1	3	4	4	4	2						3
G. davidsonii (*Ophiostoma davidsonii*)						1				2	3	2	2	1	3	
Grosmannia sp. J-5 (*O. europhioides*)	3					2	2			1	4	4	2	4	3	
Grosmannia sp. J-6 (*Ophiostoma* sp. D)	2	2	3	3	2							2	3			
Grosmannia sp. J-7 (*Ophiostoma* sp. P)		2		1	1	2	3			2	2					
Graphilbum microcarpum (*Ophiostoma* sp. V)	1					1		3	1							3
Gra. rectangulosporium (*Ophiostoma* sp. M)		3	2	1	2	1	2	2	3							2
Ophiostoma nikkoense (*Ophiostoma* sp. S)						1		3								3
O. piceae		2			1	1	2	2		2				3	1	3
O. subalpinum	4	4	4	3	4	3	4	2	4	2	2	2	4			
Ophiostoma sp. (*Ophiostoma* sp. K)									1	1						

[a] Beetle population collected at the same time from *Abies mariesii* (Am), *A. veitchii* (Av) and *A. homolepis* (Ah).
[b] Frequency of occurrence of each fungus. 1: less than 10%, 2: 10–40%, 3: 40–70%, 4: more than 70%, based on the data presented in Yamaoka et al. (2004a).
[c] Fungal name in parenthesis was one shown in Yamaoka et al. (2004a).

Zipfel, Z.W. de Beer & M.J. Wingf. and *Grosmannia* sp. J-5 were associated mainly with *Dryocoetes hectographus* Reitter and *D. autographus* (Ratzeburg); and *O. subalpinum* and *Gra. rectangulosporium* were associated mainly with *P. proximus* and *Cryphalus montanus* Nobuchi (Yamaoka et al. 2004a). *Grosmannia aoshimae* were closely associated with *P. proximus* and *Grosmannia* sp. J-6 were associated with *C. montanus* (Yamaoka et al. 2004a). *Graphilbum microcarpum* and *O. nikkoense* were associated mainly with *Dryocoetes striatus* Eggers or the host plant, *A. homolepis*.

2.2. *Ophiostomatoid fungi from Japanese larch*

The eight-spined *Ips* beetle, *Ips subelongatus* (Motschulsky), is an important bark beetle in Japan because it can damage Japanese larch [*Larix kaempferi* (Lamb.) Carrière] plantations (Koizumi 1990). The ophiostomatoid fungi associated with *I. subelongatus* in Japan were investigated by Aoshima (1965) and Yamaoka et al. (1998, 2009). *Ips subelongatus* was formerly considered to be conspecific to *I. cembrae* (Heer), which infests European larch (*L. decidua* Mill.) in Europe. At least 12 species of ophiostomatoid fungi have been reported (Table 2). Among them, *E. fujiensis* (M.J. Wingf., Yamaoka & Marin) Z.W. de Beer, T.A. Duong & M.J. Wingf. (= *Ceratocystis fujiensis* M. J. Wingf., Yamaoka & Marin) (Fig. 2), *G. laricis* (Van der Westh., Yamaoka & M.J. Wingf.) Zipfel, Z.W. de Beer & M.J. Wingf., and *O. breviusculum* Chung, Yamaoka, Uzunovic & Kim (Fig. 3) were reported only on Japanese larch in Japan.

Endoconidiophora fujiensis in association with *I. subelongatus* was first reported as *C. laricicola* Redfern & Minter (Yamaoka et al. 1998), which is associated with *I. cembrae* in Europe (Redfern et al. 1987). Marin et al. (2005) recognized that the Japanese isolates of *C. laricicola* were distinguishable from European isolates based on DNA sequence comparisons and were treated as a distinct taxon, *C. fujiensis*. As mentioned above, De Beer et al. (2014) divided *Ceratocystis* s. l. into seven genera based on a phylogenetic analysis using the sequence data of three genomic regions. *Ceratocystis fujiensis* was transferred to the genus *Endoconidiophora*.

Grosmannia laricis was first described by Van der Westhuizen et al. (1995) as *O. laricis* Van der Westh., Yamaoka & M.J. Wingf. Zipfel et al. (2006) conducted a molecular phylogenetic study, and separated *Grosmannia* from *Ophiostoma*. This species was transferred to the *Grosmannia* and was recognized as a member of the *G. piceiperda* complex based on a molecular phylogenic analysis (Linnakoski et al. 2012). This species has curved ascospores instead of cucullate ascospores, which are shared by several members of the *G. piceiperda* complex.

Ophiostomatoid fungi associated with other bark beetles infesting Japanese larch were reported by Tokumasu (2009). Revised information for these fungi together with unpublished data are shown in Table 2. There were several undescribed species. A few species of *Grosmannia* were isolated from bark beetles infesting Japanese larch. Ando et al. (2016) conducted molecular phylogenetic and morphological studies of the Japanese *G. piceiperda* complex. *Grosmannia* spp., reported in Yamaoka et al. (2009) and Tokumasu (2009), are now recognized as distinct species. *Grosmannia* sp. J-3 was obtained from samples collected in Nikko, Tochigi Prefecture, and *Grosmannia* sp. D and J-4 from Nagao Prefecture.

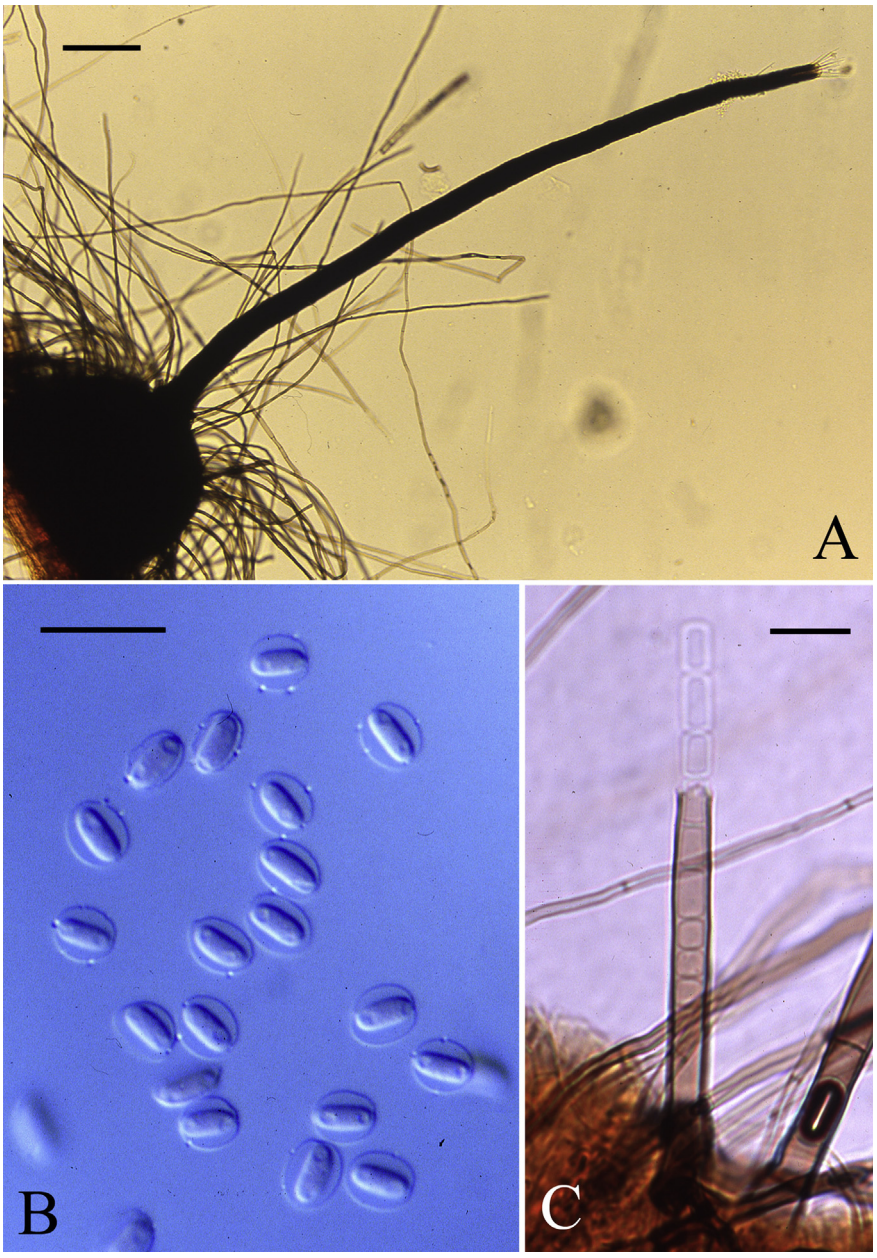

Fig. 2 – *Endoconidiophora fujiensis* [Adapted from Yamaoka et al. (1998) except for Fig. 4C]. A: Perithecium. B: Ascospores. C: *Thielaviopsis* asexual morph [Adapted from Yamaoka et al. (1999)]. *Bars*: A 100 μm, B 10 μm, C 15 μm.

Ophiostoma breviusculum was described as a new species of the *O. piceae* complex by Chung et al. (2006). It is distinguishable from *O. piceae* (Münch) Syd. & P. Syd. based on morphology, molecular phylogenetic analysis and mating experiments. Later, Yamaoka et al. (2009) reported isolates that were morphologically and ecologically indistinguishable from those in Chung et al. (2006), but which were unable to interbreed. Further studies are required to determine the taxonomic status of this population.

Ophiostoma cf. *piceae* was recognized as a distinct species, *O. sugadairense* J. Li, Yamaoka & Masuya (Li et al. 2017). It was distinguished from other species of the *O. piceae* complex by a molecular phylogenetic analysis and its synnema morphology. This species was closely associated with the bark beetle, *Polygraphus kisoensis* Niijima.

Ophiostoma sp. F (Yamaoka et al. 2009) is morphologically similar to *G. abieticola*, but clearly distinguishable from it based on its larger perithecia. The asexual state of the species is similar to that of *Leptographium taigense* Linnakoski, Z.W. de Beer & M.J. Wingf. (Linnakoski et al. 2012). Precise morphological comparisons and molecular phylogenic studies are required to determine the taxonomic position of this fungus.

Ophiostoma brunneo-ciliatum Math.-Käärik was frequently associated with *I. subelongatus*. Aoshima (1965) also reported that *O. brunneo-ciliatum* was a dominant fungus associated with bark beetles attacking Japanese larch, including *I.*

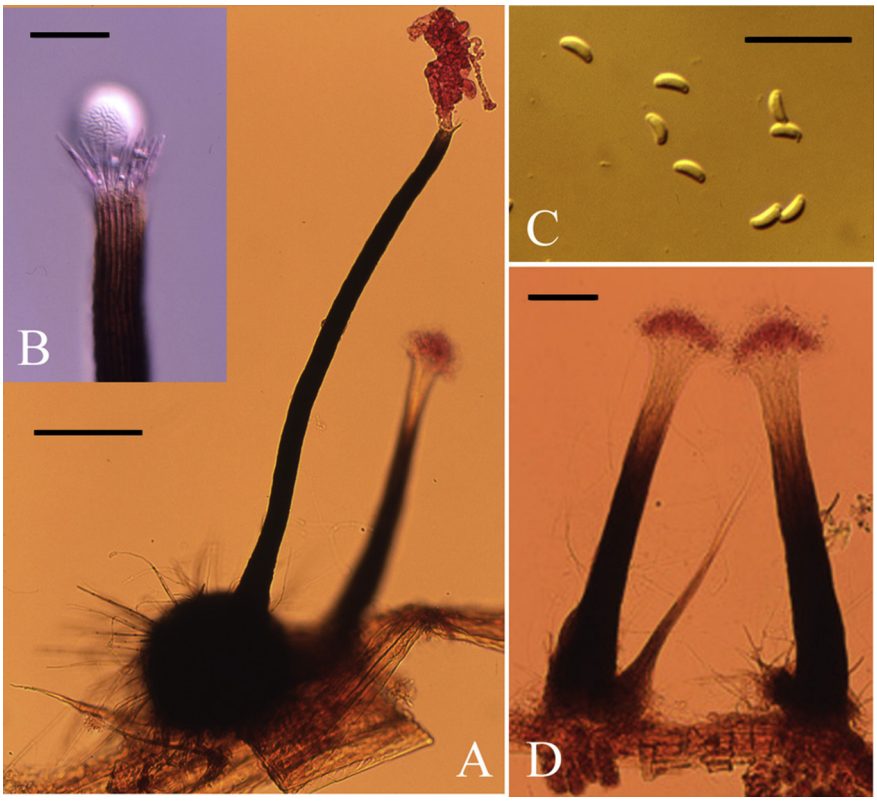

Fig. 3 – *Ophiostoma breviusculum* [Adapted from Yamaoka et al. (2006)]. A: Perithecium. B: Ostiolar hyphae at the tip of neck. C: Ascospores. D: *Pesotum* asexual morph. *Bars*: A, D 40 μm, B 30 μm, C 10 μm.

subelongatus (as *I. cembrae*). In Europe, it is associated with *I. sexdentatus* (Boerner) on pine (Mathiesen-Käärik 1953) and *I. cembrae* on larch (Stauffer et al. 2001). Linnakoski et al. (2016) conducted phylogenic analyses of European isolates reported as *O. brunneo-ciliatum* and *O. clavatum* Math., together with related species, using the ITS, βT, elongation factor 1-α (TEF-1α), and CAL genes. The results revealed that this species complex; i.e., the *O. clavatum*-complex, included seven cryptic species. Among them, two species, *O. brunneo-ciliatum* and *O. pseudocatenulatum* Jankowiak, R. Linnakoski & Z.W. de Beer, were related to *Ips cembrae* in *Larix decidua*. Molecular phylogenic analyses of the Japanese isolates are required to determine their identity.

Graphilbum fragrans (Math.-Käärik) Z.W. de Beer, Seifert & M.J. Wingf. was reported on bark beetles infesting Japanese red pine (*Pinus densiflora* Siebold et Zucc.) as *Pesotum fragrans* (Math.-Käärik) Okada & Seifert by Masuya et al. (2009). This species was considered an asexual state of *Ophiostoma* species, but not a member of the *O. piceae* complex by Okada et al. (1998) and Harrington et al. (2001) based on 18S rDNA and ITS sequences, respectively. It was treated as a member of *Graphilbum* by De Beer et al. (2013b) based on molecular phylogenic analyses of the LSU and ITS of rDNA.

Ceratocystis coerulescens (Münch) Bakshi was also isolated from *I. subelongatus* (as *I. cembrae*) by Aoshima (1965) and Yamaoka et al. (2009). This fungus was first described as *E. coerulescens* by Münch (1907), and was long treated as one species. However, it was subsequently segregated into five species distinguishable by their morphology and isozyme variation (Wingfield et al. 1997; Harrington and Wingfield 1998). Japanese isolates were identified as *C. coerulescens* s. str. (Yamaoka et al. 2009), which was classified as one species of *Endoconidiophora* by De Beer et al. (2014).

Ceratocystiopsis minuta (Siem.) H.P. Upadhyay & W.B. Kendr. (= *O. minutum* Siem.) was isolated from *I. subelongatus* by Yamaoka et al. (1998). It is discussed in the following section, together with isolates from spruce.

2.3. *Ophiostomatoid fungi from spruce (Picea spp.)*

Ips typographus japonicus seriously damages Yezo spruce [*Picea jezoensis* (Siebold et Zucc.) Carrière] and Sachalin spruce [*P. glehnii* (F. Schmidt) Mast.] in Hokkaido, Japan. The ophiostomatoid fungi associated with beetles infesting *Picea* spp. trees have been investigated extensively (Aoshima 1965; Yamaoka et al. 1997). Ten species were recorded by Yamaoka et al. (1997) (Table 3), most of which were identical to those associated with *I. typographus* infesting *P. abies* (L.) Karst. in Europe (Masuya et al. 2013). Both Solheim (1986) and Yamaoka et al. (1997) reported *G. europhioides* was frequently associated with bark beetles in Europe and Japan. However, the Japanese population was subsequently classified as *G. aenigmatica* (K. Jacobs, M.J. Wingf. & Yamaoka) Zipfel, Z.W. de Beer & M.J. Wingf. (= *O. aenigmaticum* K. Jacobs, M.J. Wingf. & Yamaoka), which produce perithecia with a shorter neck. It is a member of the *G. piceiperda* complex and has been reported only in Japan (Linnakoski et al. 2012; Ando et al. 2016). When Yamaoka et al. (1997) reported *G. europhioides* associated with

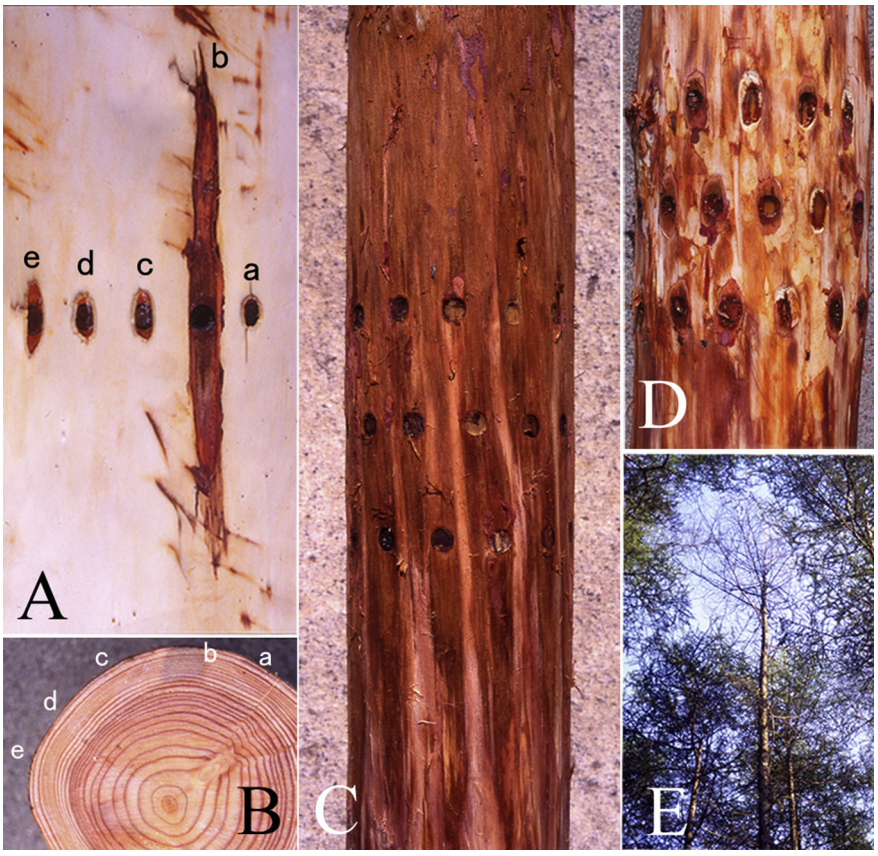

Fig. 4 – Lesions and symptoms produced after inoculation with ophiostomatoid fungi [Adapted from Yamaoka et al. (1998)]. A: Necrotic lesions produced in the inner bark around the sites of inoculation with the control (a), *Endoconidiophora fujiensis* (b), *Ophiostoma piceae* (c), *O. brunneo-ciliatum* (d), and *Grosmannia laricis* (e). B: Cross-section of a stem of an inoculated larch tree. A blue-stained area was produced in sapwood after inoculation with *E. fujiensis* (b). C: Dead inner bark tissues after inoculation at multiple points of a Japanese larch tree with *E. funiensis*. D: Lesions produced after inoculation at multiple points of a Japanese larch tree with *O. piceae*. E: Symptoms of a Japanese larch tree after inoculation at multiple points with *E. funiensis*.

I. typographus japonicus, two isolates, YCC-111 and YCC-242, were indicated as reference isolates. The results of the molecular phylogenetic analysis by Ando et al. (2016) demonstrated that isolate YCC-111 was *G. aenigmatica* and isolate YCC-242 was *Grosmannia* sp. D, which was another species of the *G. piceiperda* complex that produced perithecia with short necks. The European *G. europhioides* was different from that in North America (Linnakoski et al. 2012; Ando et al. 2016).

Endoconidiophora polonica was first described as *O. polonicum* Siemaszko (Siemaszko 1939), with a *Leptographium* asexual morph in Europe. This information led to confusion in the identification of the Japanese population of *C. polonica*, which had a *Thielaviopsis* asexual morph. Visser et al. (1995) reported that the European population has a *Thielaviopsis* rather than a *Leptographium* asexual morph. A rDNA analysis revealed that this fungus belongs to the genus *Ceratocystis*. This species was transferred to the genus *Endoconidiophora* by De Beer et al. (2014).

Tokumasu (2009) reported ophiostomatoid fungi associated with bark beetles in *Picea* spp. logs in Sugadaira, Nagano Prefecture, central Honshu, including several undescribed species (Table 3). Species of the *G. piceiperda* complex are now identified as *G. piceiperda* D (Ando et al. 2016), which is distributed in Russia (Linnakoski et al. 2012).

Grosmannia davidsonii was isolated from *Dryocoetes autographus* and *Hylurgops transbaicalicus* Eggers in *Picea* spp. Based on the studies mentioned above, *G. davidsonii* was almost always vectored by *D. autographus* and *D. hectographus*. Ohtaka et al. (2002a) reported the isolation of this species mainly from *D. hectographus* infesting *A. veitchii* in wave-regenerated forests in central Honshu.

Ceratocystiopsis species were isolated from *I. typographus japonicus* in Yezo spruce and *I. subelongatus* in Japanese larch. These isolates were identified as one species, *Ceratocystiopsis minuta* by Yamaoka et al. (1997, 1998). Plattner et al. (2009) conducted a molecular phylogenic analysis of putative strains of *Cop. minuta* from Europe, Japan, and North America using the β-tubulin gene, and the ITS and LSU regions of rDNA. The Japanese isolates were segregated into two clades together with the European isolates. The YCC-139 isolate from *I. typographus japonicus* in Yezo spruce, which clustered with several Polish isolates, was considered to be *Cop. minuta* (Reid and Hausner 2010). Isolates from *I. subelongatus* in Japanese larch were treated as *Ceratocystiopsis* sp. 2 (Plattner et al. 2009; De Beer and Wingfield 2013). Further studies are required to determine the taxonomic position of the Japanese isolates. *Ceratocystiopsis* spp. produced smaller perithecia on older

Table 2 – Frequencies of occurrence of ophiostomatoid fungi isolated from bark beetles infesting Japanese larch.

Species	*Ips subelongatus*[a]								*Dryocoetes baicalicus*	*D. hectographus*		*D. pini*	*Polygraphus kisoensis*	*P. meakaensis*
	No. 1 Ka	No. 2 Sa	No. 3 Iw	No. 4 Ya	No. 5 Ka	No. 6 Ka	No. 7 Ni	No. 8 Ni	Ni[b]	Ni	Su[b]	Su	Su	Ni
Ceratocystiopsis cf. *minuta*	2[c]	2	1				3							
Cop. neglecta (*Ophiostoma* sp. S-2)											1	1	1	
Endoconidiophora coerulescens (*Ceratocystis coerulescens*)[d]							2	2						
E. fujiensis (*C. fujiensis*)		3			4	3	3							
Grosmannia davidsonii										2	2			
G. laricis	3	4	3	3	4	4	3	3	3	4		1		
G. piceiperda D (*Grosmannia* sp. 3)											3			
Grosmannia sp. J-4 (*Grosmannia* sp. L2, *Grosmannia* sp. 4)	2										2	4	1	
Grosmannia sp. J-3 (*Grosmannia* sp. L2)								2	2					
Ophiostoma brunneociliatum	3	4	2	2	4	3	3	3	2				2	1
O. floccosum	1						2	1	1		2			2
O. piceae	4	4	4	2	4	4	4	4	2	4		2		3
Ophiostoma sp. F	2	2	1	3		2	2	3	2	4	2	1		1
O. cf. *breviusculum*	2							2	4		3	4	2	
O. cf. *piceae*											2		4	
Sporothrix cf. *stenocerus*													1	
Sporothrix sp. 61 (*Ophiostoma* sp. 61)														2
Graphilbum fragrans	3	0	2	2			2	4	4	1	2	4	1	1

[a] Data on *Ips subelongatus* were based on Table 2 in Yamaoka et al. (2009). Fungal name in parenthesis was one shown in Yamaoka et al. (2009).
[b] Ni: Samples were collected at Nikko, Tochigi, Japan (unpublished data), Su: Samples were collected at Sugadaira, Nagano, Japan [based on data in Tokumasu (2009)].
[c] Frequency of occurrence of each fungus. 1: less than 10%, 2: 10–40%, 3: 40–70%, 4: more than 70%, based on the data presented in Yamaoka et al. (2009).
[d] Fungal name in parenthesis was one shown in Yamaoka et al. (2009).

Table 3 – Frequency of occurrence of ophiostomatoid fungi from bark beetles infesting spruce (*Picea* spp.).

Species	*Ips typographus japonicus*[a]	*Cryphalus fulvus*	*Cryphalus* sp.	*Dryocoetes autographus*			*Hylurgops transbaicalicus*		*Polygraphus* sp.	*Tomicus piniperda*
	Pj1[b]	*Pg6*	*Pk4*	*Pk4*	*Pk5*	*Pg6*	*Pk5*	*Pg6*	*Pk4*	*Pk4*
Ceratocystiopsis sp. 2 (*Ceratocystiopsis minuta*)	2[c]									
C. neglecta (*Ophiostoma* sp. S-2)			1							
Endoconidiophora polonica (*Ceratocystis polonica*)[d]	2									
Grosmannia aenigmatica (*Ophiostoma aenigmaticum*)	2									
G. cucullata (*O. cucullatum*)	1									
Grosmannia davidsonii				2	2	2	2	2		
G. koreana (*O. koreanum*)		1				3		2		
G. penicillata (*O. penicillatum*)	4									
G. piceiperda D (*Grosmannia* spp. 2, 3 and 5)		2	2	2	4	2	4	4	2	4
Ophiostoma ainoae	4									
O. bicolor	3									
O. brunneo-ciliatum							1			2
O. floccosum			2	3	2		2		2	4
O. cf. *japonicum*	2						1			
O. piceae	4	2			4	4	4	4		
O. cf. *piceae*			1							
Ophiostoma sp. No. 8	1									
Ophiostoma sp. S-1						2	1			
Ophiostoma sp. F (*Ophiostoma* sp. Y)		3	2			2	1		2	
Leptographium sp. 4					2		2			
Leptographium spp.		1								

[a] Data on *Ips typographus japonicus* were based on Table 1 in Yamaoka et al. (1997).

[b] Beetle population collected at the same time from *Picea jezoensis* (*Pj*), *Pi. koyamae* (*Pk*) and *Pi. glauca* (*Pg*). *Ips typographus japonicus* were collected at Furano, Hokkaido and the other samples were collected at Sugadaira, Nagano, Japan [based on data in Tokumasu (2009)].

[c] Frequency of occurrence of each fungus. 1: less than 10%, 2: 10–40%, 3: 40–70%, 4: more than 70%, based on the data presented in Yamaoka et al. (1997) and Tokumasu (2009).

[d] Fungal name in parenthesis was one shown in Yamaoka et al. (1997) or Tokumasu (2009).

galleries and grew more slowly on artificial medium, so that the presence of the fungus was overlooked and its isolation might not be successful.

The species reported as *Ophiostoma* sp. S-2 by Tokumasu (2009) was identified as *Ceratocystiopsis neglecta* (R. Kirschner & Oberw.) Z.W. de Beer & M.J. Wingf. based on morphology and molecular phylogenic analyses (unpublished data by Kuroda et al.). This species was first described as *O. neglectum* R. Kirschner & Oberwinkler (Kirschner and Oberwinkler 1999). It has cucullate instead of falcate ascospores, which are more common among *Ceratocystiopsis* species. A DNA sequence analysis by De Beer and Wingfield (2013) confirmed that *O. neglectum* in Europe belonged to *Ceratocystiopsis*. European isolates were associated with four bark beetles infesting Norway spruce. Japanese isolates originated from several bark beetle species in Japanese larch and *Picea koyamae* Shiras. Their frequencies of isolation were low, and the major associated bark beetle species were therefore unclear.

3. Pathogenicity of ophiostomatoid fungi against host trees

3.1. *Virulence of ophiostomatoid fungi*

As mentioned above, some ophiostomatoid fungi are pathogenic towards their host plants. Generally, the reappearance of the original symptoms after inoculation using infection processes similar to those in nature is the criterion used to determine the pathogen of a particular plant disease. Pathogenic ophiostomatoid fungi are introduced to their host plants through wounds produced by feeding and gallery construction (Webber and Gibbs 1989) by beetles. The introduction of fungi under the bark results in the production of necrotic lesions in the phloem around beetle galleries and colonization of sapwood, possibly resulting in death of the tree (Harrington 1993b). Thus, the virulence of the fungi was evaluated by measuring lesion sizes and monitoring the mortality of host plants after inoculation. The separate evaluation of these two parameters is important (Paine et al. 1997). However, producing wounds for inoculation following gallery construction by bark beetles is problematic.

Several methods can be used to compare the virulence of the ophiostomatoid fungi associated with bark beetles. One such method is to measure the size of the lesions produced around the inoculation points on the tree trunk. For example, bark discs (4 mm–1 cm diam) are removed using a cork borer at intervals of about 3 cm in a horizontal ring at a height of 1.5 m on the trunk. Inoculum discs of the same size taken from fungal colonies or bark discs harboring fungi are placed into the wounds. This inoculation method is useful for inducing damage to the tree and ensuring an identical inoculum size. The wound is slightly larger than the entrance hole made by bark beetles, but is almost identical to that of their nuptial chambers.

The inoculated trees are felled at 2–3.5 mo after inoculation, and the size of the lesions formed in the inner bark around the inoculation points, together with the sizes of the stained and dried zones of sapwood, are measured. The necrotic lesions that form around the inoculation points on the inner bark spread mainly longitudinally, and only slightly in the tangential direction, and hence are ellipsoid to fusiform in shape. The lengths of lesions differ according to the fungus inoculated. Additionally, a wedge-shaped dry zone of sapwood with or without blue staining spreads from the inoculation point toward the border between the sapwood and heartwood. Following inoculation of Japanese larch trees with *E. fujiensis* (as *C. laricicola*), the dry zone reached the border between the sapwood and heartwood at 55 d after inoculation (Yamaoka et al. 1998) (Fig. 4). The fungi produced larger lesions and were more virulent in the dry zones.

Similar methods were used by Molnar (1965) to evaluate the virulence of *Grosmannia dryocoetidis* (W.B. Kendr. & Molnar) Zipfel, Z.W. de Beer & M.J. Wingf. (= *Ceratocystis dryocoetidis* W.B. Kendr. & Molnar) against alpine fir, by Redfern et al. (1987) to assess the virulence of *E. laricicola* against European larch, and by Solheim (1988) to determine the virulence of ophiostomatoid fungi associated with *I. typographus* against Norway spruce. Some of the fungi, such as *E. fujiensis* (Yamaoka et al. 1998), *E. laricicola* (Redfern et al. 1987) and *G. clavigera* (Solheim and Krokene 1998), produced both larger necrotic lesions in the inner bark and dry zones in the sapwood. In contrast, *E. polonica* formed larger dry zones in the sapwood, but smaller necrotic lesions in the inner bark, while *G. penicillata* (Grosmann) Goid. and *Grosmannia* spp. formed larger necrotic lesions but smaller dry zones than *E. polonica* (Solheim 1988; Yamaoka et al. 2000). The ability to kill living tissue in the inner bark and cambium might not always correlate with the ability to colonize sapwood. Weaker pathogens, such as *O. piceae*, form smaller necrotic lesions and dry zones (Yamaoka et al. 1998, 2000).

3.2. *The ability of ophiostomatoid fungi to kill trees*

To evaluate the ability of ophiostomatoid fungi to kill their host trees, the inoculation pattern is critical. Ideally, the pattern of bark beetle galleries, which are the point of entry of the fungi to sapwood tissues, should be simulated; however, this is problematic. Because tangential growth of the fungus is limited, a greater number of inoculation points (e.g., Horntvedt et al. 1983; Christiansen 1985), flaps (Yamaoka et al. 1995), or belts (Yamaguchi et al. 1991) in a horizontal circumference girdling the stem in at least one plane are required to completely colonize sapwood around the inoculation zone. The ability of the fungi to invade sapwood and disturb water conduction is important for evaluating its ability to kill its host tree (Mathre 1964; Horntvedt et al. 1983; Solheim 1988, 1992; Yamaoka et al. 1998, 2000). Some ophiostomatoid fungi associated with bark beetles have the ability to kill their host trees; e.g., *G. dryocoetidis* associated with *Dryocoetes confusus* Swaine (Molnar 1965), *E. polonica* associated with *I. typographus* (Solheim 1988) and *I. typographus japonicus* (Yamaoka et al. 2000), *E. laricicola* associated with *I. cembrae* (Redfern et al. 1987), *E. fujiensis* associated with *I. subelongatus* (Yamaoka et al. 1998), and *G. clavigera* associated with *Dendroctonus ponderosae* (Yamaoka et al. 1995).

For example, 30-y-old Japanese larch trees (about 15 cm diam at breast height: dbh) were inoculated by removing discs of bark using a cork borer (1 cm diam), at intervals of 2 cm in three horizontal rings, which were approximately 1.5 m above ground and separated by 4 cm vertically. Thus, in total, 45 bark

discs were removed from the trees. The trees inoculated with *E. fujiensis* died within 3.5 mo of inoculation (Yamaoka et al. 1998). Large necrotic lesions were formed on the inner bark of trees inoculated with *E. fujiensis* and had merged to completely girdle the trunk around the inoculation zone. The inner bark of the trees inoculated with *E. fujiensis* above the inoculation zone was dead (Fig. 4). The entire sapwood near the inoculation zone was blue-stained. Blue-stained areas of the sapwood spread more than 1 m above and below the inoculation zone. Therefore, *E. fujiensis* had the ability to kill inoculated Japanese larch trees under the above-mentioned conditions.

Christiansen (1985) inoculated Norway spruce trees with *E. polonica* by removing discs using a cork borer (5 mm diam), to create eight holes per square decimeter within 60-cm-wide belts of the stem at 90–150 cm above ground level. This dosage was lethal to Norway spruce (*Picea abies*) trees. The same inoculation method was used by Yamaoka et al. (2000) to demonstrate that *E. polonica* had the ability to kill Yezo spruce (*P. jezoensis*) trees.

In sapwood colonized by pathogenic ophiostomatoid fungi, there was no water conduction and bordered pits of tracheids were aspirated (Nelson 1934; Mathre 1964; Basham 1970). The mechanisms responsible for the blockage of water conduction were unclear. Basham (1970) mentioned some of the substances occluding the pits; e.g., products of decomposition of parenchyma cells by the fungi (Nelson 1934), extracellular polysaccharides produced by the fungi (Mathre 1964), and resin globules (Bramble and Holst 1940). However, Basham (1970) observed no obstructing materials in tracheids or bordered pits. Kuroda (2005) suggested that secondary metabolites; i.e., a resinous substance and other water-insoluble materials, synthesized in the epithelium, and ray cells close to the infection site as a defense reaction against *C. polonica*, contribute to blockage of sap flow. She also suggested the involvement of materials that promote cavitation in sapwood.

Phytotoxins produced by ophiostomatoid fungi might contribute to the death of inoculated trees. Cerato-ulmin, a peptide produced by *O. ulmi* and *O. novo-ulmi*, caused wilting and death of elm trees (Takai 1974; Richards 1993). However, Temple et al. (1997) reported that cerato-ulmin is a hydrophobic protein that protects infectious propagules from desiccation; therefore, cerato-ulmin production might not be related to the virulence of the fungi. Toxic substances in cell-free culture filtrates of the pathogen were reported to cause wilting of plants and cuttings (Richards 1993). Thus, the contribution of cerato-ulmin and other phytotoxins should be re-examined and the mechanisms of wilting clarified. In addition, polysaccharides (McWain and Gregory 1972) and phenolic metabolites (DeAngelis et al. 1986) produced by ophiostomatoid fungi might be related to wilting of host trees.

4. Conclusion

Many ophiostomatoid fungi are associated with bark beetles. Some species have specific associations with particular bark beetles and/or are specific to a host tree species, while others are common to several species of bark beetles and/or host trees. However, recent studies have demonstrated the presence of cryptic species worldwide (e.g., Johnson et al. 2005; Linnakoski et al. 2012; Ando et al. 2016). Advances in molecular techniques have facilitated the detection of cryptic species. De Beer and Wingfield (2013) have reported several species complexes that were formerly considered to be one species. Wingfield et al. (2016) reported the importance of accurate identification of the microorganisms (e.g., ophiostomatoid fungi) associated with forest insects (e.g., bark beetles) involved in devastating diseases of trees using appropriate molecular markers. More precise taxonomic identification will enhance our understanding of the relationships among ophiostomatoid fungi, bark beetles, and plants, and the coevolution and species differentiation of ophiostomatoid fungi.

Ploetz et al. (2013) reviewed destructive tree diseases associated with ambrosia and bark beetles. They proposed several factors associated with the development of new bark beetle-associated problems; i.e., biogeographical shifts, pathogen-vector shifts, and host shifts. Wingfield et al. (2016) divided symbiosis between forest insects and microorganisms causing devastating diseases of trees into four categories based on the native/exotic status of the species involved. The findings revealed that microorganisms that are important drivers of forest pestilence were introduced along with their associated insects (both insect and microorganism were exotic) in most cases, but there were many examples of novel associations in which one was native and the other exotic. The authors emphasized the importance of further studies of the associations between forest insects and microorganisms to predict novel associations, since the insects and microorganisms involved in the novel associations were previously associated with phylogenetically related and/or ecologically similar organisms. In addition to research on the novel associations between ophiostomatoid fungi and bark beetles involved in devastating diseases of trees, those on the associations in native populations, including under endemic conditions, are warranted.

Evaluation of the virulence of plant pathogenic fungi is important in phytopathology and forest pathology. The mechanisms underlying the death of inoculated trees are at present unclear, but phytotoxins and/or secondary metabolites, particularly hydrophobic substances, produced by the pathogenic fungi may be involved. Differences in virulence towards trees might influence the relationship of ophiostomatoid fungi with bark beetles; therefore, the differentiation of ophiostomatoid fungus species is vital.

Organisms other than ophiostomatoid fungi are also associated with bark beetles. For example, yeasts; e.g., *Ogataea pini* (Holst) Y. Yamada, M. Matsuda, K. Maeda & Mikata (= *Pichia pini* (Holst) Phaff) and *Nakazawaea holstii* (Wick.) Y. Yamada, K. Maeda & Mikata (= *Hansenula holstii* Wick.) (Shifrine and Phaff 1956; Endoh 2012; etc.), basidiomycetes; e.g., *Entomocorticium* spp. (Whitney et al. 1987; Hsiau and Harrington 2003), *Phlebiopsis gigantes* (Fr.) Jülich (Tsuneda et al. 1993; Hibbett and Thorn 2001), *Gloeocystidium ipidophilum* Siem. (Siemaszko 1939), and *Chionosphaera cuniculicola* R. Kirschner, Begerow & Oberw. (Kirschner et al. 2001), a discomycete, *Pezizella chapmanii* H. S. Whitney & A. Funk (Whitney and Funk 1977), and others (e.g., Whitney 1982; Harrington 2005; Hofstetter et al. 2015) are closely associated

with bark beetles. Pholetic mites might vector ophiostomatoid fungi (Bridges and Moser 1983; Moser and Bridges 1986), and predators of bark beetles might carry the basidiomycete, *Cryptoporus volvatus* (Peck) Shear (Borden and McClaren 1970). Bark beetles and their galleries are useful for biological studies, and collaborations with biologists in other research fields are required to clarify the interrelationships among these organisms.

Disclosure

The author declares no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country where they were performed.

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