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Ophiostomatoid fungi associated with *Ips cembrae* in Japan and their pathogenicity to Japanese larch

Yuichi Yamaoka[1], Michael J. Wingfield[2], Masashi Ohsawa[3] and Yoshio Kuroda[1]

[1] Institute of Agriculture and Forestry, University of Tsukuba, Tsukuba, Ibaraki 305–8572, Japan
[2] Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa
[3] Yamanashi Forestry and Forest Products Research Institute, Masuhomachi, Minamikomagun, Yamanashi 400–0502, Japan

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Six ophiostomatoid fungi, i.e., *Ceratocystiopsis minuta*, *Ceratocystis laricicola*, *Ophiostoma brunneo-ciliatum*, *O. laricis*, *O. piceae* and *Ophiostoma* sp., were isolated from the galleries of *Ips cembrae* on Japanese larch (*Larix kaempferi*) logs in central Honshu, Japan. Japanese larch trees approximately 30 yr old were inoculated with all six fungi. *Ceratocystis laricicola* produced the largest lesions on the inner bark around the inoculation point and the largest dry zone in the sapwood. Furthermore, *C. laricicola* was the only fungus associated with *I. cembrae* that had the ability to kill Japanese larch, death occurring in 30-yr-old trees within 3.5 mo after inoculation.

Key Words——*Ceratocystiopsis minuta*; *Ceratocystis laricicola*; inoculation experiment; *Ips cembrae*; *Ophiostoma* spp.

Ips cembrae (Heer) infests trees and recently cut logs of larch (*Larix* spp.) in Europe and Asia (Crooke and Bevan, 1957; Nobuchi, 1974; Redfern et al., 1987; Koizumi, 1990, 1994). The insect also damages plantations of European larch (*L. decidua* Miller) in the United Kingdom (Crooke and Bevan, 1957; Redfern, 1989) and Japanese larch (*L. kaempferi* (Lamb.) Carr.) in Japan (Koizumi, 1990). Redfern et al. (1987) described *Ceratocystis laricicola* Redfern & Minter from *I. cembrae* in Scotland and showed that the fungus has a high degree of virulence.

In Japan, Aoshima (1965) isolated six species of ophiostomatoid fungi, some of them undescribed, from *I. cembrae* infesting Japanese larch: *Ceratocystis coerulescens* (Münch) Bakshi, *C. jezoensis* Aoshima nom. nud., *C. macrospora* Aoshima nom. nud., *Ophiostoma brunneo-ciliatum* Mathiesen-Käärik, *O. olivaceum* Mathiesen, and *O. piceae* Münch. Amongst them, *O. piceae* was shown to be pathogenic (Maeto et al., 1991; Yamaguchi, 1993, 1995) and was considered to be responsible for the mortality of larch trees infested by *I. cembrae* (Maeto et al., 1991; Yamaguchi, 1995). Only *O. piceae* was used in the inoculation study, and it was not compared with the other ophiostomatoid fungi associated with *I. cembrae*.

Although *C. laricicola* Redfern u Z. Minter is known as the dominant fungus associated with *I. cembrae* on introduced larch plantations in Scotland (Redfern et al., 1987), its occurrence on native stands of larch, in either Europe or Japan, has not been reported. *Ips cembrae* in Scotland is thought to have been introduced from Continental Europe and it is logically assumed that *C. laricicola* was introduced with this insect. Aoshima (1965) invalidly described *C. jezoensis* from larch in Japan and it is probable that this fungus is the same as *C. laricicola*.

One of the aims of this study was to characterize the ophiostomatoid fungi from larch in Japan and to compare and contrast these findings with previous investigations. Based on a previous study of fungi associated with *I. typographus* L. f. *japonicus* Niijima in Japan (Yamaoka et al., 1997) and the study of Redfern et al. (1987), we have hypothesized that *C. laricicola* would be the most virulent and important fungal associate of *I. cembrae* in Japan. A further aim of this study was to prove this hypothesis. If correct, this would be in contrast to the findings of Maeto et al. (1991) and Yamaguchi (1995) who did not isolate *C. laricicola* and who suggested that *O. piceae* is an important and virulent associate of *I. cembrae*.

Materials and Methods

Isolation of fungi Disks approximately 5 cm thick and strips of bark (about 10 × 20 cm) including galleries of *I. cembrae* were cut on 20 July 1989 from Japanese larch logs left in plantations (about 1,500 m a.s.l.) after thinning in Experimental Forests in Yatsugatake (EFY), Agricultural and Forestry Research Center, University of Tsukuba, Kawakami-mura, Nagano Prefecture. On 10 July 1992, similar samples were collected from a Japanese larch plantation (1,420 m a.s.l.) at the foot of Mt. Fuji, Fujiyoshida-shi, Yamanashi Prefecture. In both areas, the populations of the beetles seemed to be endemic. Samples were placed in plastic bags and transferred to the laboratory for further study. With the aid of a dissection microscope, masses of ascospores accumulating at the tips of perithecia, or masses of conidia accumulating on conidiophores were carefully lifted from these structures. This was accomplished by using a fine tungsten needle, with which the spore masses were transferred onto 1% malt extract agar (1% MA; 10 g malt extract, 15 g agar/1,000 ml distilled water) in 9-cm Petri dishes. The dishes were incubated at 20°C in the dark and the fungi were allowed to sporulate. The process of purifying and identifying cultures was similar to that described by Yamaoka et al. (1997). Cultures used for identification were grown on 2% malt extract agar, 2% malt extract Ebios agar (2% MEA; 20 g malt extract, 1 g Ebios (Brewer's yeast preparation, Tanabe), 15 g agar/1,000 ml distilled water), and 1% Pablum agar (PA; 10 g Pablum mixed cereal, 15 g agar/1,000 ml distilled water), with or without the addition of small (about 2 cm × 5 mm × 3 mm) pieces of autoclaved Japanese larch bark.

Cultures representing each distinct taxon of ophiostomatoid fungus have been deposited in the culture collection of the Laboratory of Plant Pathology and Mycology, Institute of Agriculture and Forestry, University of Tsukuba, Tsukuba, Japan and in the Japan Collection of Microorganisms (JCM). Dried specimens of these cultures have also been deposited with the Herbarium of the Institute of Agriculture and Forestry, University of Tsukuba (TSH).

Inoculations To assess the relative virulence of the ophiostomatoid fungi associated with *I. cembrae* on larch, eight isolates representing the six taxa (Table 1) were selected. These included two isolates each of *C. laricicola* and *O. piceae* and one each of the other four taxa. Isolates YCC-273 (*C. laricicola*) and YCC-299 (*O. piceae*) were acquired from Japanese larch logs collected in Experimental Forests in Yatsugatake, and all other isolates were from Mt. Fuji.

Experiment I. In this experiment, all six fungi were tested for their relative virulence. Each fungus was grown on 2% MA in 9-cm Petri dishes at 20°C for 2 wk. Disks of bark (1 cm in diam and 2–3 mm in thickness), which were punched out with a cork borer from healthy Japanese larch bark after removing the scaly outermost layer of bark, were autoclaved for 20 min at 121°C and added to the plates. The plates were incubated under the same conditions for an additional 3 wk. Perithecia and/or conidiophores of the test fungi were produced on the bark discs, which were used as inoculum discs. For controls, sterilized bark discs were placed on 2% MA and these were incubated at 20°C until use.

Four Japanese larch trees approximately 30 yr old (15.5 to 18 cm DBH) were selected in a plantation (about 1,500 m a.s.l.) in the EFY. Each tree was inoculated with all test fungi and controls on 30 May 1995. Bark discs (1 cm in diam) were removed using a cork borer at intervals of about 3 cm in a horizontal ring at a height of 1.5 m (Fig. 1a). Inoculum discs or control discs were placed into the wounds and covered with vaseline followed by parafilm and adhesive tape. The trunks of the trees were then wrapped with nylon sheet from ground level to a height of 2 m to prevent invasion by bark beetles.

Two of the inoculated trees were felled on 24 July 1995 (55 d after inoculation). Three 1-m logs were cut from the bottom of each tree and to the laboratory for examination. Bark was peeled from the bolts and the size of lesions formed in the inner bark around the inoculation points was measured. The logs were then cut into disks at about 10-cm intervals above and below the inoculation points. Staining and dried zones of sapwood were observed on the surface of each disc. Small pieces of wood were cut from the stained sapwood and transferred onto 2% MA plates to confirm the presence of the inoculated fungi. The remaining two trees were felled on 11 September 1995 (104 d after inoculation). Lesions on the inner bark, as well as staining and the presence of dry zones of sapwood, were observed using the technique described above.

Experiment II. In this experiment, six trees approximately 30 yr old (13–15 cm dbh) in the same stand of the experimental forest as in the first inoculation experiment were subjected to three different treatments on 30 May 1995. The aim here was to compare the relative virulence of *C. laricicola* and *O. piceae*. Two trees each were inoculated with inoculum discs of *C. laricicola* YCC-285 or *O. piceae* YCC-295, or with sterile bark discs as controls. Inoculation was achieved by removing discs of

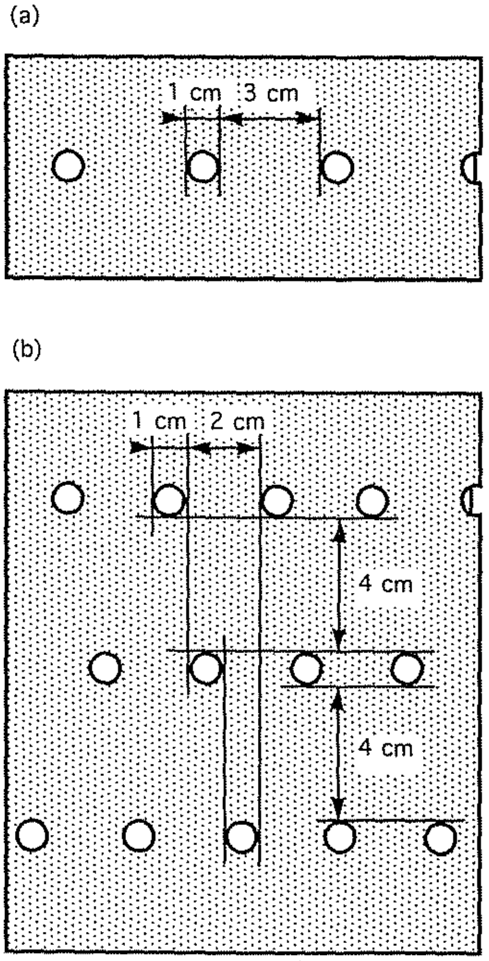


Fig. 1. Patterns of inoculation in Experiment I (a) and II (b).

bark with a cork borer (1 cm in diam), at intervals of 2 cm in three horizontal rings, which were approximately 1.5 m above ground and separated by 4 cm vertically (Fig. 1b). Thus a total of 45 bark discs were removed from each tree with 15 cm dbh. The inoculations were covered as described above.

Development of the external symptoms of inoculated trees was monitored at intervals of 2–3 wk. Two trees inoculated with *C. laricicola*, and one of each of the trees inoculated with *O. piceae* or sterile bark discs were felled on 11 September 1995 (104 d after inoculation). One-meter bolts were cut from the boles from the ground level up to 3 m above ground and transferred to the laboratory for further study. Lesions formed in the inner bark around the inoculated area as well as stained and dry zones of sapwood were measured. Symptoms of the remaining two trees inoculated with *O. piceae* or sterile bark discs were observed until early November, when needles of uninoculated normal larch trees in the same stand turned yellow. They were felled on 7 November 1995 (161 d after inoculation) and treated as described above.

Results and Discussion

Isolation of fungi

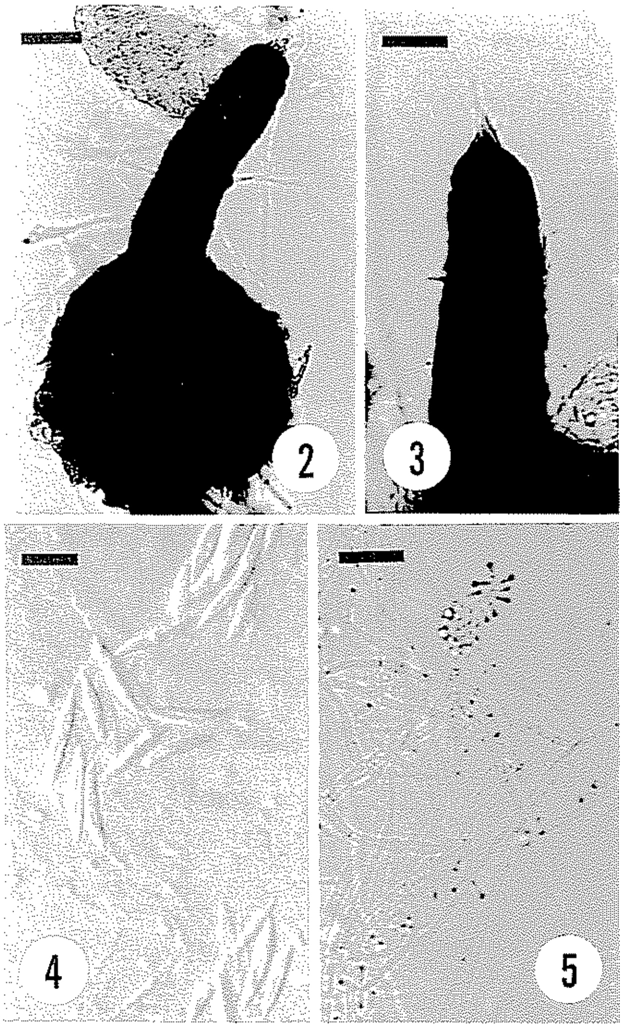
Six distinct species of ophiostomatoid fungi were isolated from Japanese larch infested with *I. cembrae*. These are as follows.

Ceratocystiopsis minuta (Siem.) Upadhyay & Kendrick, Mycologia **67**: 800. 1975. Figs. 2–5
≡*Ophiostoma minutum* Siem., Planta Pol. **7**: 23. 1939.
≡*Ceratocystis minuta* (Siem.) Hunt, Lloydia **19**: 49. 1956.

Morphological characteristics of the fungus isolated in the present study were almost the same as those of *C. minuta* associated with Yezo spruce (*Picea jezoensis* (Sieb. & Zucc.) Carr.) infested by *I. typographus* f. *japonicus* in Japan (Yamaoka et al., 1997). One exception was that the perithecia of the present fungus were uniformly brown to dark brown (Fig. 2), while those of the fungus associated with Yezo spruce had lighter bases when produced on the bark placed on the surface of agar (Yamaoka et al., 1997). They also fitted well with the description for *C. minuta* (Hunt, 1956; Upadhyay, 1981).

Ceratocystiopsis minuta is known to be associated with Norway spruce infested by *I. typographus* in Europe (Siemaszko, 1939; Mathiesen, 1950; Kotýnková-Sychrová, 1966; Käärik, 1975; Solheim, 1986, 1993; Harding, 1989), and other spruce and pine trees associated with various bark beetles including *Dendroctonus* spp. and *Ips* spp. in North America (Davidson, 1942; Wright and Cain, 1961; Robinson, 1962; Griffin, 1968; Olchowecki and Reid, 1974; Upadhyay, 1981). In Japan, Yamaoka et al. (1997) provided the first record of a *Ceratocystiopsis* sp., and the present study is the second report of *C. minuta* associated with a bark beetle in Japan.

Living cultures deposited: YCC-293, isolate from a gallery wall of *I. cembrae* in *L. kaempferi* at the foot of Mt. Fuji, Fujiyoshida-shi, Yamanashi, Japan (FMF). Collected on 10 July 1992 by Y. Yamaoka (YY), M. J. Wingfield (MJW), and M. Ohsawa (MO); YCC-294, isolate from a gallery wall of *I. cembrae* in *L. kaempferi* at FMF. Collected on 10 July 1992 by YY, MJW, and MO.

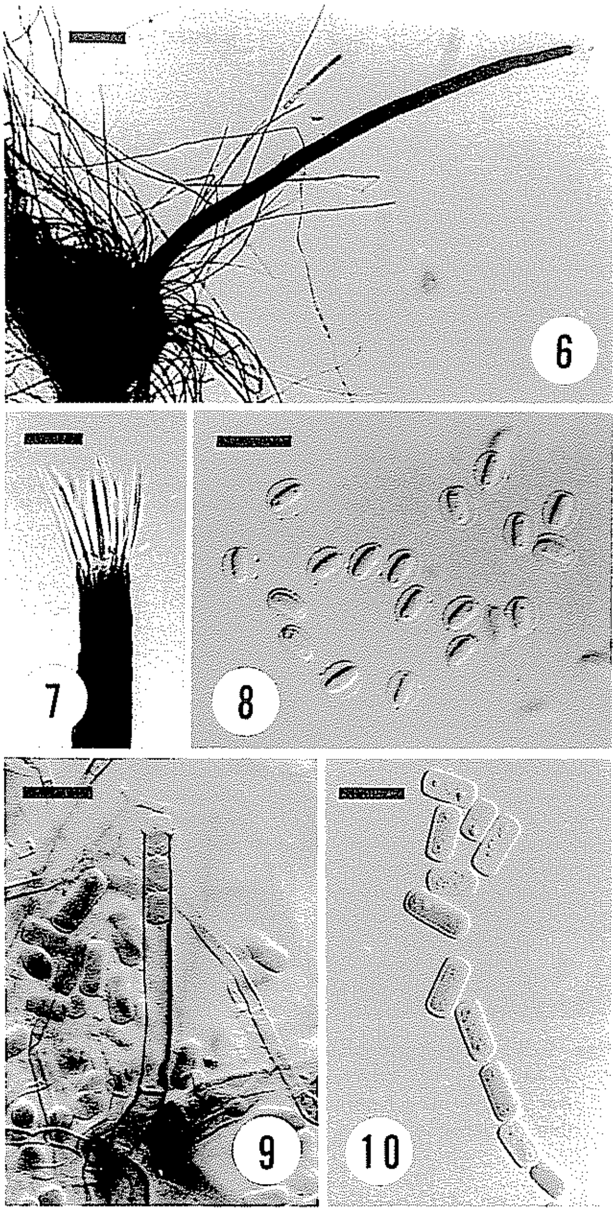


Figs. 2–5. *Ceratocystiopsis minuta*.
2. Ascocarp (bar, 20 μm). 3. Top of neck (bar, 15 μm). 4. Ascospores (bar, 5 μm). 5. *Hyalorhinocladiella* anamorph (bar, 15 μm).

Dried specimens: TSH-C127, dried culture YCC-293 grown on 1% Pablum agar with pieces of autoclaved bark of *L. kaempferi* (PAB) at 20°C; TSH-C128, dried culture YCC-294 grown on PAB at 20°C.

Ceratocystis laricicola Redfern & Minter, in Redfern et al., Plant Pathol. **36**: 468. 1987. Figs. 6–10

Perithecia of this fungus were produced superficially both on the surface of the agar medium and on bark placed on the surface of the agar. The bases of perithecia (Fig. 6) were black, globose to subglobose, 145–300 μm in diam, ornamented by dark brown septate hyphae. Perithecial necks were slender, black,



Figs. 6–10. *Ceratocystis laricicola*.
6. Ascocarp (bar, 100 μm). 7. Top of neck (bar, 20 μm). 8. Ascospores (bar, 10 μm). 9. *Chalara* anamorph (bar, 15 μm). 10. Conidia (bar, 15 μm).

540–1,150 μm long; terminating ostiolar hyphae (Fig. 7) aseptate, hyaline, straight, 14–22, slightly divergent, 32–67 μm long. Ascospores (Fig. 8) ellipsoid, 4.8–6.0 × 2.4–2.8 μm, surrounded by hyaline sheaths, oval in face view, 4.8–6.4 × 3.6–4.4 μm, and orange-section shaped in side view. Conidiophores (Fig. 9) mononematous, cylindrical or slightly tapered, pale brown to brown, composed of 2–8 cells, 94–274 μm long, including terminal conidiogenous cell, and 4–8 μm wide at the broadest part. Conidiogenous cells phialidic, cylindrical or lageniform, and 56–100 × 4–8 μm. Conidia (Fig. 10) hyaline, oblong, barrel-shaped or ellipsoidal, 6–15 × 3.5–6 μm.

This fungus was first described by Redfern et al. (1987) as being associated with *I. cembrae* attacking introduced European larch in Scotland. It has recently been suggested that *C. laricicola* might be conspecific with *C. polonica* (Siem.) C. Moreau (Visser et al., 1995; Harrington et al., 1996; Witthuhn et al., 1998), which is a common associate of *I. typographus* in Europe (Siemaszko, 1939; Mathiesen, 1950, 1951; Mathiesen-Käärik, 1953, 1960; Käärik, 1975; Solheim, 1986, 1993; Harding, 1989; Furniss et al., 1990; Viiri and von Weissenberg, 1995). The present fungus was virtually identical in morphological characteristics to *C. laricicola* described by Redfern et al. (1987), except for the size of ascospores, which were about 4 × 2 μm excluding the sheath. It was also morphologically indistinguishable from *C. polonica* associated with *I. typographus* f. *japonicus* (Yamaoka et al., 1997) except that diameters of the perithecial bases of the Japanese isolates (145–300 μm) were slightly smaller than those of *C. polonica* (200–390 μm), and the length of conidiophores (94–274 μm including terminal conidiogenous cells) were longer than those of *C. polonica* (74–160 μm).

Aoshima (1965) described a *Ceratocystis* species associated with *I. typographus* f. *japonicus* which appears to be the same as *C. polonica* (Yamaoka et al., 1997). He named it *C. jezoensis,* but this description was not valid, since it was not formally published. He also noted that this fungus was associated with *I. cembrae* in Japan. It is apparent that he considered the fungus associated with *I. cembrae* to be conspecific with that associated with *I. typographus* f. *japonicus*. *Ceratocystis laricicola* and *C. polonica* are very similar and morphologically indistinguishable. Visser et al. (1995) were unable to distinguish these species by comparison of sequence data from the ITS region of the ribosomal DNA. These data have also recently been confirmed in a more comprehensive study, at the molecular level, of *Ceratocystis* spp. from conifers (Witthuhn et al., 1998). Similarly, isolates of *C. laricicola* grouped closely with those of *C. polonica* in a comparison of *Ceratocystis* spp. based on isozymes (Harrington et al., 1996). However, in the latter study, isolates of *C. laricicola* could be distinguished from *C. polonica,* and the fungi have very different hosts and distinct vectors. The fungi might thus represent distinct taxa that have recently undergone speciation and, thus, have not developed distinct morphological characteristics. For the present, we believe that they are best treated as distinct.

Living cultures deposited: YCC-273, isolate from a gallery wall of *I. cembrae* in *L. kaempferi* at Experimental Forests in Yatsugatake, Agricultural and Forestry Research Center, University of Tsukuba, Kawakami-mura, Nagano, Japan (EFY). Collected on 20 July 1989 by YY; YCC-285[1] (JCM9810), isolate from a gallery wall of *I. cembrae* in *L. kaempferi* at FMF. Collected on 10 July 1992 by YY, MJW, and MO.

Dried specimens: TSH-C106, dried culture YCC-285

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[1] The same culture as C745 used in Harrington et al. (1996).

grown on PAB at 20°C; TSH-C132, dried culture YCC-273 grown on 2% malt extract Ebios agar with pieces of autoclaved bark of *L. kaempferi* (MEAB) at 20°C.

Ophiostoma brunneo-ciliatum Mathiesen-Käärik, Meddnl. St. Skogsforskningsinst. **43**: 21. 1953. Figs. 11–15
≡*Ceratocystis brunneo-ciliata* (Math.-K.) Hunt, Lloydia **19**: 32. 1956.

This fungus produced perithecia superficially both on the surface of the agar medium and on the bark placed on the agar. The bases of perithecia (Fig. 11) were black, globose to subglobose, 150–270 μm in diam and necks slender, black, 810–1,080 μm long. Ostiolar hyphae (Fig. 12) 12–20, brown, spirally curved, 24–56 μm long. Ascospores (Fig. 13) ossiform or rectangular in side view, 3.2–4.8 × 1.2–1.6 μm, including hyaline sheaths. Morphological characteristics of the fungus were consistent with the descriptions of *O. brunneo-ciliatum* (Mathiesen-Käärik, 1953; Hunt, 1956; Aoshima, 1965).

Ophiostoma brunneo-ciliatum resembles *O. ainoae* H. Solheim and *O. clavatum* Mathiesen, since all these fungi have brown, spirally curved ostiolar hyphae at the tips of perithecial necks and *Graphium*-like anamorphs. They can, however, be distinguished from each other based on ascospore form (Hunt, 1956; Solheim 1986). *Ophiostoma brunneo-ciliatum* has ascospores that are ossiform or rectangular in side view, whereas *O. ainoae* has ascospores that are cylindrical in side view, and *O. clavatum* has ascospores that are orange-section shaped. *Ophiostoma brunneo-ciliatum* is also distinguished from *O. ainoae* by its larger perithecia.

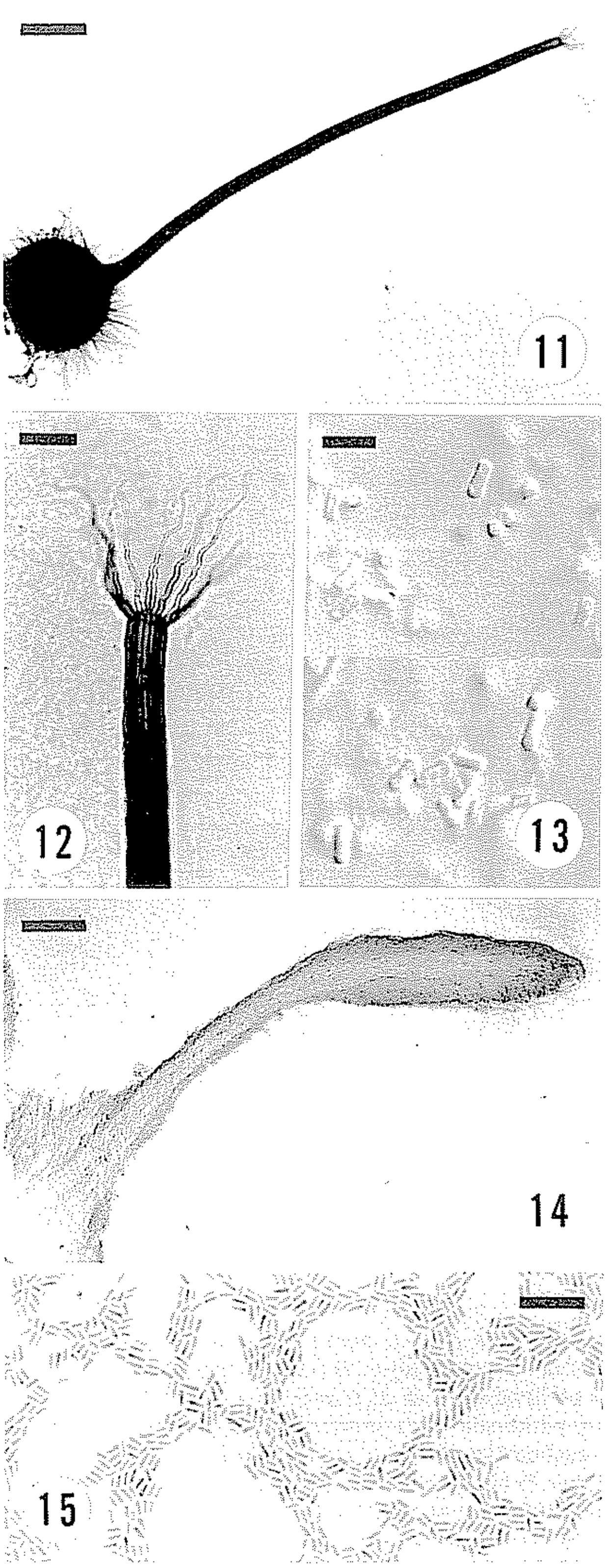
Ophiostoma brunneo-ciliatum was first described from Sweden, where it is associated with the bark beetle *I. sexdentatus* Boern (Mathiesen-Käärik, 1953). Aoshima (1965) reported that *O. brunneo-ciliatum* was one of the dominant fungi associated with *I. cembrae*. This fungus is weakly pathogenic to Scots pine (*Pinus sylvestris* L.) (Lieutier et al., 1989).

Living cultures deposited: YCC-276, isolate from the wall of an *I. cembrae* gallery in *L. kaempferi* at EFY. Collected on 20 July 1989 by YY; YCC-287 (JCM9811), isolate from the wall of an *I. cembrae* gallery in *L. kaempferi* at FMF. Collected on 10 July 1992 by YY, MJW, and MO.

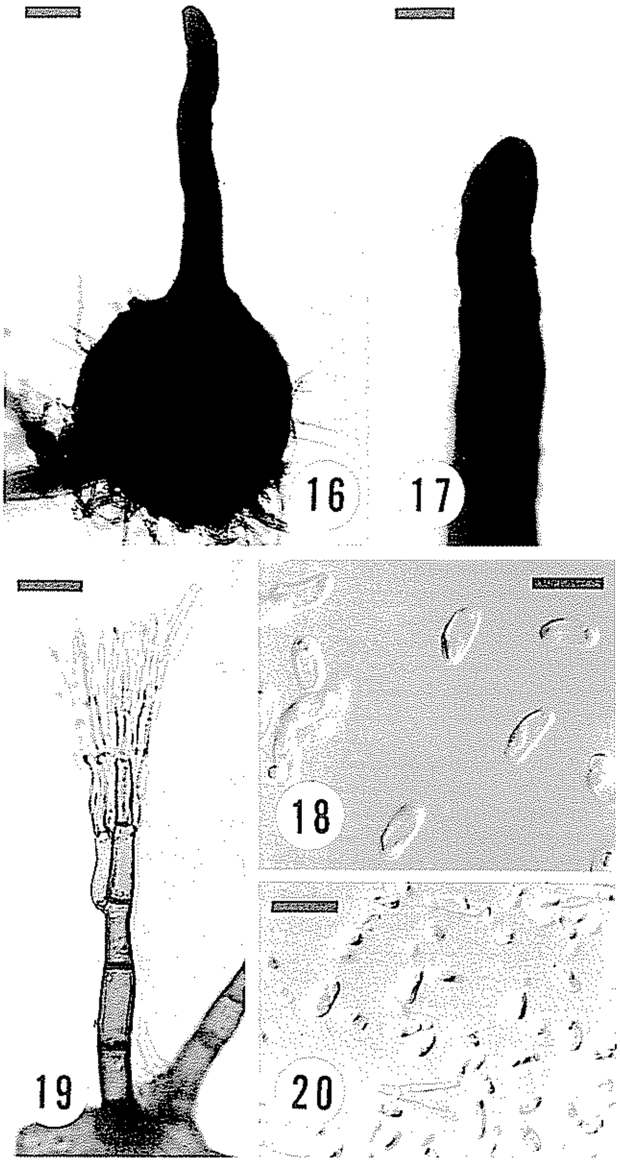
Dried specimens: TSH-C112, dried culture YCC-287 grown on PAB at 20°C; TSH-C138, dried culture YCC-276 grown on MEAB at 20°C.

Ophiostoma laricis K. Van der Westhuizen, Y. Yamaoka & M. J. Wingfield, Mycol. Res. **99**: 1336. 1995. Figs. 16–20

This fungus was first described by Van der Westhuizen et al. (1995) based on specimens collected at Mt. Fuji. It was also isolated from samples collected at Experimental Forests in Yatsugatake, Nagano, and is not known from countries other than Japan. Perithecia of *O. laricis* resemble *O. europhioides* (Wright & Cain) H. Solheim and *O. huntii* (Robins.-Jeff.) de Hoog & Scheffer. The ascospores of *O. laricis* are curved and surrounded by a thick uniform sheath (Fig. 18), while those of *O.*



Figs. 11–15. *Ophiostoma brunneo-ciliatum*.
11. Ascocarp (bar, 100 μm). 12. Top of neck (bar, 20 μm). 13. Ascospores (bar, 5 μm). 14. *Graphium* anamorph (bar, 200 μm). 15. Conidia (bar, 15 μm).



Figs. 16–20. *Ophiostoma laricis*.
16. Ascocarp (bar, 50 μm). 17. Top of neck (bar, 20 μm). 18. Ascospores (bar, 10 μm). 19. *Leptographium* anamorph (bar, 15 μm). 20. Conidia (bar, 15 μm).

europhioides and *O. huntii* have cucullate ascospores. Van der Westhuizen et al. (1995) described the lengths of perithecial necks of *O. laricis* as 400–1,320 μm when the perithecia were produced on host tissue or in the presence of host tissue and on autoclaved pieces of *Pinus patula* Schl. & Cham. on 2% MEA plates. The present fungus, however, produced perithecia with shorter necks (200–370 μm long) when the cultures were grown on 1% Pablum agar amended with pieces of autoclaved bark of Japanese larch (PAB) at 20°C (Fig. 16).

Aoshima (1965) described a *Ceratocystis* species (*C. macrospora* nom. nud.) associated with *I. cembrae* that was morphologically very similar to *O. laricis*. *Ceratocystis macrospora* was not valid, since it was not formally published.

Living cultures deposited: YCC-277, isolate from the wall of *I. cembrae* gallery in *L. kaempferi* at EFY. Collected on 20 July 1989 by YY; YCC-289 (JCM9812), isolate from the wall of *I. cembrae* gallery in *L. kaempferi* at FMF. Collected on 10 July 1992 by YY, MJW, and MO.

Dried specimens: TSH-C117, dried culture YCC-289 grown on PAB at 20°C; TSH-C140, dried culture YCC-277 grown on MEAB at 20°C.

Ophiostoma piceae (Münch) H. & P. Sydow, Ann. Mycol. **17**: 43. 1919.
≡*Ceratostomella piceae* Münch, Naturw. Z. Land- u. Forstw. **5**: 547. 1907.
≡*Ceratocystis piceae* (Münch) Bakshi, Trans. Br. Mycol. Soc. **33**: 113. 1950.

Ophiostoma piceae isolated in this study had the same morphological characteristics as the fungus isolated from *I. typographus* f. *japonicus* in Hokkaido (Yamaoka et al., 1997). It was also very similar to the description of *O. piceae* by Hunt (1956) and Upadhyay (1981), except that the bases of perithecia were larger (up to 280 μm in diam). Detailed descriptions of Japanese isolates of *O. piceae* have been presented previously (Nishikado and Yamauti, 1935; Aoshima, 1965).

Aoshima (1965) reported that *O. piceae* was one of the most common fungi associated with *I. cembrae*. This fungus has been isolated from Yezo spruce (Tochinai and Sakamoto, 1934; Aoshima, 1965; Yamaoka et al., 1997), Japanese red pine (*Pinus densiflora* Sieb. & Zucc.) (Nishikado and Yamauti, 1935; Aoshima, 1965), beech (*Fagus crenata* Blume) (Aoshima and Hayashi, 1953; Aoshima, 1965), and various other conifers and hardwoods (Otani, 1988).

Brasier and Kirk (1993) separated *O. piceae* into two intersterile mating groups, one from hardwood sources (OPH) and one from conifers (OPC). They further suggested that OPH probably represents *O. querci* (Georgevitch) Nannf.=*Ceratostomella quercus* Georgevitch (1927) and OPC the original *O. piceae*=*Ceratostomella piceae* sensu Münch (1907). Halmschlager et al. (1994) supported their findings by morphological investigation of synnematal size and RAPD analysis of DNA. The fungus associated with *I. cembrae* in the present study is thus considered to be *O. piceae*. However, Japanese isolates of *O. piceae* from conifer and hardwood sources must still be examined using mating tests and molecular comparisons to clarify their taxonomic position.

Living cultures deposited: YCC-299, isolated from the wall of an *I. cembrae* gallery in *L. kaempferi* at EFY. Collected on 20 July 1989 by YY; YCC-301, isolate from the wall of an *I. cembrae* gallery in *L. kaempferi* at EFY. Collected on 20 July 1989 by YY.

Dried specimens: TSH-C156, dried culture YCC-301 grown on PAB at 20°C; TSH-C157, dried culture YCC-299 grown on PAB at 20°C.