Comparison of *Ophiostoma huntii* and *O. europhioides* and description of *O. aenigmaticum* sp. nov.

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Ophiostoma europhioides and *O. huntii* are two very similar fungi known to cause blue-stain of timber. Both species have been isolated from pine and spruce in Canada and are characterized by *Leptographium* anamorphs and hat-shaped ascospores. The aim of this study was to compare isolates of *O. huntii* and *O. europhioides* from various parts of the world. These isolates were compared morphologically using light and scanning electron microscopy. Sexual compatibility was determined using mating studies. *O. huntii* isolates from the different geographical areas were similar to each other but distinct from *O. europhioides*. A new *Ophiostoma* species, *O. aenigmaticum*, is described for the Japanese isolates; *Leptographium aenigmaticum*, its anamorph, is described as a new species.

Ophiostoma Syd. & P. Syd., *Ceratocystis* Ellis & Halst, and *Ceratocystiopsis* H. P. Upadhyay & W. B. Kendr. are collectively treated in the *Ceratocystis sensu lato* complex or more generally grouped in the so-called ophiostomatoid fungi. Controversy has surrounded the taxonomy of this group of fungi since their discovery at the end of the last century (Halsted, 1890; Sydow & Sydow, 1919; Upadhyay & Kendrick, 1975). Some taxonomists have considered *Ophiostoma* and *Ceratocystis* to be synonyms (Bakshi, 1951; Upadhyay, 1981); others have treated them as separate taxa based on morphological and physiological characteristics (Arx, 1952; de Hoog & Scheffer, 1984; Harrington, 1987). Recent analysis of ribosomal DNA sequence data has shown that *Ophiostoma* and *Ceratocystis* are unrelated (Hausner, Reid & Klassen, 1993*a*; Spatafora & Blackwell, 1994), while *Ophiostoma* and *Ceratocystiopsis* might be considered synonymous (Hausner *et al*., 1993*b*).

Ophiostoma europhioides (E. F. Wright & Cain) H. Solheim and *O. huntii* (Rob.-Jeffr.) de Hoog & R. J. Scheff. are two species commonly associated with blue-stain of conifer timber. *Ophiostoma europhioides* was first described by Wright & Cain (1961) from pine in Canada. *O. huntii*, which is morphologically similar to *O. europhioides*, was also described from pine in Canada (Robinson-Jeffrey & Grinchenko, 1964). Although these fungi are very similar, Robinson-Jeffrey & Grinchenko (1964) distinguished them on ascospore and perithecial dimensions. This distinction based on ascospore size was also made by Olchowecki & Reid (1974) in their survey of ophiostomatoid fungi in Manitoba. Both species are found wide-spread throughout the United States (Davidson & Robinson-Jeffrey, 1965). *O. europhioides* was also reported from Norway and Germany (Davidson, Francke-Grosmann & Käärik, 1967; Solheim, 1986). Upadhyay (1981) considered *O. europhioides* to be a synonym of *O. piceaperdum* (Rumbold) Arx. Solheim (1986), however, treated these two species as distinct as he had not seen material of *O. piceaperdum* and did not wish to comment further on their synonymy. In the original description of *O. piceaperdum*, Rumbold (1936) noted that the ascospores of this species are ellipsoid in contrast to the hat-shaped ascospores of *O. europhioides*. The true morphological nature of *O. piceaperdum* is, therefore, uncertain. In this study we follow the taxonomic treatment of Solheim (1986) for *O. europhioides*.

In recent years, new isolates of both *O. huntii* and *O. europhioides* from various parts of the world have become available for study. This has prompted us to reconsider the morphological characteristics of both species. The aim of this study was thus to compare isolates of these fungi from the different geographical areas with each other and in the context of their original descriptions.

MATERIALS AND METHODS

Morphology. A list of isolates used in this study, as well as details regarding their origin, is presented in Table 1. Cultures were grown at 35 °C on 2% malt extract agar (MEA) (20 g malt extract, Biolab; 20 g agar, Biolab; 1 l distilled water) to which pieces of autoclaved sapwood of *Pinus patula* Schlecht. & Cham. were added after cooling. When developed, characteristic fruiting structures were taken from the wood on the agar, mounted in lactophenol cotton blue and measured using light microscopy. Fifty measurements were made of each structure and the ranges and averages computed. For SEM examination, small pieces of agar were cut from sporulating colonies and prepared and examined according to Van der Westhuizen *et al*. (1995).

Single ascospore and conidial isolates were obtained by removing ascospore and conidial masses from the tops of

Table 1. Isolates of *Ophiostoma* species used

	Origin	Isolates*	Supplier†
O. huntii	Europe	CMW 2264; CMW 622	N. Anselmi, Italy; M. De Fatima Moniz, Portugal
	New Zealand	CMW 1006; CMW 1003; CMW 1004; CMW 1010; CMW 1005; CMW 1007; CMW 1009; CMW 1011; CMW 1015	M. Mackenzie, formerly of Forest Research Institute, Rotorua; New Zealand
	Australia	CMW 181; CMW 182; CMW 183; CMW 184; IMI 5551	J. Simpson IMI
	U.S.A.	CMW 654 (CBS 399.77); CMW 486 (CBS 153.75)	CBS
		CMW 1756	C. Bertagnolli
		C113; C139	T. C. Harrington
O. aenigmaticum	Japan	CMW 2200; CMW 2313; CMW 2310; CMW 2199; CMW 231	Y. Yamaoka
O. europhioides	Europe	CMW 477 (CBS 229.83); CMW 446 (CBS 153.65)	CBS
		CMW 3314; CMW 3313; CMW 3312	T. Kiristis
	U.S.A.	CMW 452 (CBS 275.65); CMW 479 (CBS 444.69)	CBS

* CMW, culture collection of M. J. Wingfield, C, culture collection of T. C. Harrington; CBS, Centraalbureau voor Schimmelcultures; IMI, culture collection of the International Mycological Institute.

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mature perithecia or conidiophores with a dissecting needle, and transferring them to 1 ml sterile water in Eppendorf tubes. The Eppendorf tubes were shaken using a vortex mixer, and the contents then poured onto the surface of 2% MEA plates to germinate. After 24–48 h, single germinating ascospores or conidia observed under a dissection microscope were picked from the agar surface using a sterile hypodermic needle, transferred to clean MEA-plates, and incubated at 25°.

Mating experiments. Ten single ascospore cultures from Australian isolates of *O. huntii* were paired in every possible combination, and mating strains thus identified. Single ascospore isolates were subsequently made from the progeny of such crosses, and back-crossed to the parental mating strains. From the latter crosses, mating strains were obtained that exhibited reliable sexual crossings. Single conidial or ascospore isolates from all other collections, including single ascospores of *O. europhioides*, were then paired with these tester strains.

RESULTS

Comparison of Ophiostoma huntii *isolates*

Ophiostoma huntii produces conidiophores, each with two primary branches, very sparsely in culture. This was evident in all the isolates used in this study. When present, they were generally found on the aerial mycelium and not on the agar or associated with the pine tissue, and all the isolates produced similar sized obovoid conidia with truncate ends (Table 2). Isolates of *O. huntii* from the U.S.A., Australia and New Zealand all produced similar perithecia in abundance. Perithecial neck length were in the range 350–1500 μm and the base sizes were 130–350 μm (Table 2). All isolates produced hat-shaped ascospores in the range 3–5 × 1·8–3 μm (Table 2). It was not possible, therefore, to distinguish among isolates based on either conidial or ascospore characteristics.

Comparison between Ophiostoma huntii *and* Ophiostoma europhioides

Morphologically, isolates of *O. huntii* and *O. europhioides* were very similar (Table 3). Both have *Leptographium* anamorphs and obovoid conidia and only minor differences were found in the perithecial sizes and ascospores. The Japanese isolates, however, initially identified as *O. europhioides* (Yamaoka *et al.*, 1997), had significantly shorter perithecial neck lengths than those found in the cultures accepted as *O. huntii* and *O. europhioides* herein, and the hat-shaped ascospores of the Japanese isolates also had more elongated brims. But significantly, *O. europhioides* and the Japanese isolates produced abundant conidiophores, while these were lacking in *O. huntii*. *Ophiostoma huntii* strains also had distinctly serpentine mycelium which was less obvious in either *O. europhioides* or the Japanese isolates (Table 3).

The sexual systems of *O. huntii* and *O. europhioides* appear to be significantly different. *Ophiostoma huntii* is heterothallic, and single ascospore isolates did not produce perithecia. But both single ascospore and conidial isolates from New Zealand, Europe, U.S.A. and Australia induced perithecial formation

Table 2. Comparison of ecological and morphological characteristics of *Ophiostoma huntii* isolates from different geographic regions

	Isolates*			
	U.S.A.	Europe	New Zealand	Australia
Conidial (µ)	3·1–6·8 × 1·8–3·1	3·7 × 1·8–3·7	4·3–9·3 × 1·8–3·1	3·7–8 × 1·8–3·1
Conidiophore length (µm)	99–208	108–561	68–186	99–200
Perithecial neck length (µm)	384–1200	N.O.†	350–1500	152–464
Perithecial base size (µm)	184–344	N.O.	160–300	208–320
Ascospore size	3–5 × 1·8–2·5	N.O.	3·1–5 × 1·8–3·1	3·1–5 × 1·8–2·5
Ascospore shape	Hat-shaped	N.O.	Hat-shaped	Hat-shaped

* Isolates used were as listed in Table 1.
† Not observed.

Table 3. Comparison of *O. huntii*, *O. europhiodes* and *O. aenigmaticum**

	Ophiostoma huntii	*O. europhiodes*	*O. aenigmaticum*
Region	U.S.A., Australia, New Zealand, Europe	Japan, Norway, U.S.A., Germany	Japan
Host	*Pinus* & *Picea* spp.	*Pinus* & *Picea* spp.	*Picea jezoensis*
Vector	*Dendroctonus ponderosae, Ips pini* and *Tomicus piniperda*	*Dryocoetus* spp., *Pityogenes* spp. and *Ips typographus*	*I. typographus japonicus*
Thallism	Heterothallic	Homothallic	Homothallic
Conidial size (µm)	3–13 × 1·8–3·7	3·7–7·4 × 1·3–3·1	3·7–9·3 × 1·8–3·1
Conidiophore length (µm)	68–562	59–285	53–241
Perithecial neck length (µm)	350–1500	304–912	111–310
Perithecial base width (µm)	160–344	136–288	142–254
Ascospore size (µm)	3–5 × 1·8–3·1	4·3–5·6 × 1·8–3·7	3·7–5 × 1·8–2·5
Ascospore shape	Hat-shaped with short brims	Hat-shaped with short brims	Hat-shaped with elongated brims

* Morphological characters are a combined average of the measurements for individual isolates.

when paired with mating tester strains generated from Australian isolates. In contrast, single ascospore isolates of both *O. europhioides* and the Japanese isolates, initially tentatively identified as *O. europhioides*, consistently formed perithecia in culture, and are thus homothallic. Sexual compatibility is, therefore, an important distinguishing characteristic between the groups of isolates considered in this study.

Based on these results we conclude that the isolates from Japan, although morphologically similar to both *O. huntii* and *O. europhioides* (Table 2), represent a previously undescribed species. This new taxon is described as follows.

Ophiostoma aenigmaticum K. Jacobs, M. J. Wing. & Yamaoka sp. nov.

Peritheciales atrae, abundante ornatione hyphali, colla atra, cylindrica apicali exigua angustatione versus apices. *Asci* prototunicati, hyalini, evanescentes. *Ascosporae* aseptatae, hyalinae, cucullatae cum a latere visae sunt et investitae in vagina, 4–5 ($\bar{x}$ = 4·2) × 1·8–2·5 ($\bar{x}$ = 2) µm.

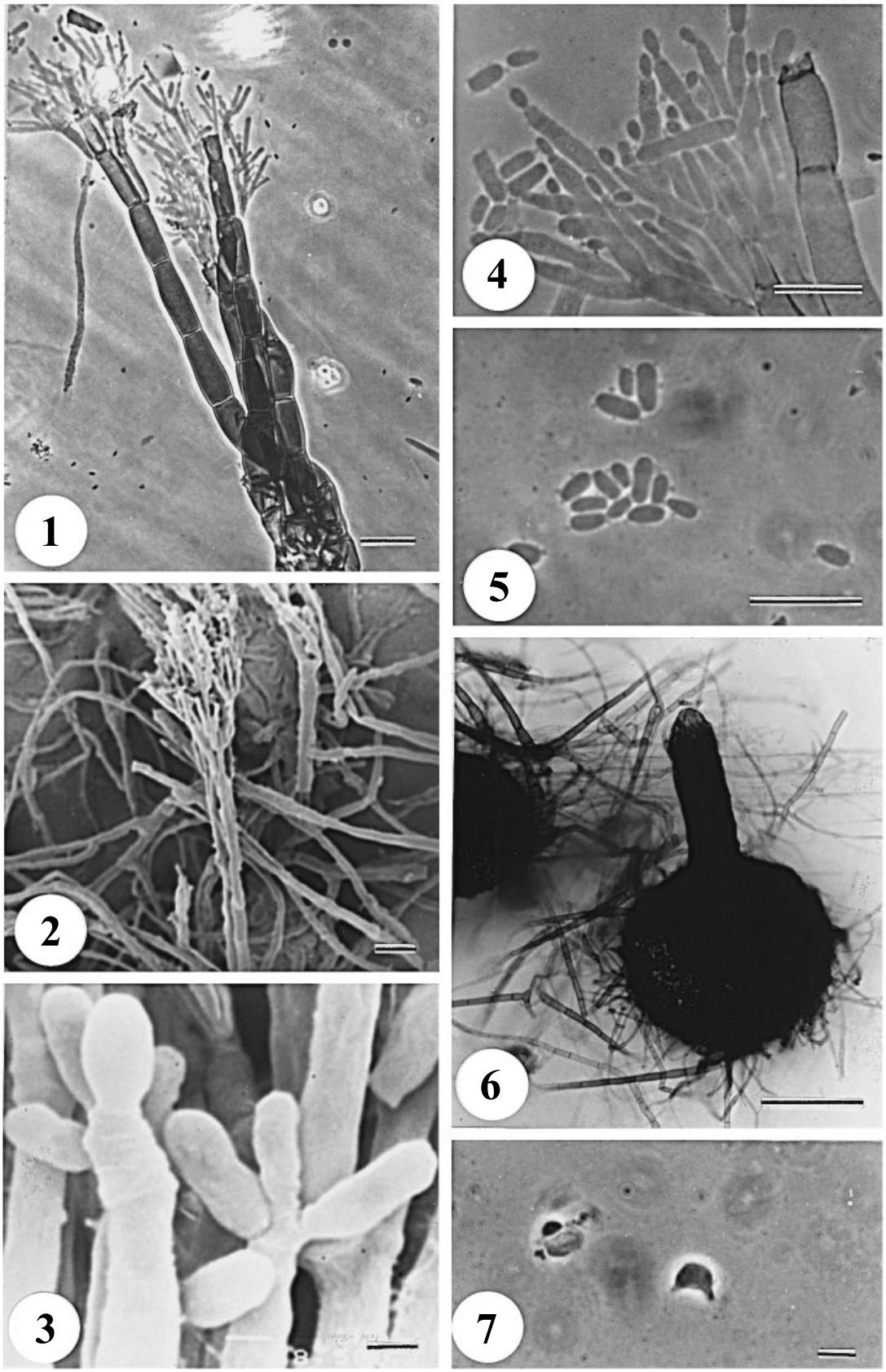
Perithecia produced superficially on 2% MEA in the presence of the host tissue. *Perithecial bases* black, globose and smooth walled, with abundant hyphal ornamentation, 143–254 ($\bar{x}$ = 204) µm diam., necks dark brown to black, cylindrical with a slight apical taper towards the apices, smooth, 117–310 ($\bar{x}$ = 180) µm long, 37–99 ($\bar{x}$ = 56) µm above globose bases, 19–43 ($\bar{x}$ = 28) µm wide at the apex, ostiolar hyphae absent (Figs 6; 8*a*). *Asci* prototunicate, hyaline, evanescent. *Ascospores* aseptate, hyaline, cucullate in side view and invested in a sheath, 4–5 ($\bar{x}$ = 4·2) × 1·8–2·5 ($\bar{x}$ = 2) µm (Figs 7, 8*b*).

Specimens examined: Cultures on 2% malt extract agar, isolated from *Picea jezoensis* Niijima infested with *Ips typographus japonicus*, Hokkaido, Japan, June 1990, Y. Yamaoka, PREM 54870 (CBS 501.96). Holotype. Paratypes: isolated from *P. jezoensis* infested with *I. typographus japonicus*, Hokkaido, Japan, June 1990, Y. Yamaoka, PREM 54871 (CBS 502.96), PREM 54872 (CBS 503.96), PREM 54873 (CBS 504.96).

Leptographium aenigmaticum K. Jacobs, M. J. Winf. & Yamaoka sp. nov.

Resistit densissimo soluto 'cycloheximide'. *Conidiophora*, exorientia directe ex mycelio, erecta, macronematosa, mononematosa, levia, olivacea vel subbrunnea, rhizoidea absentia. *Apparatus conidiogenus*: seriebus duobus vel quattuor ramorum cylindricorum; duo rami primarii. *Conidia* hyalina, aseptata, obovoidea vel allantoidea, rotundatis apicibus et truncatis basibus, 4–9 (x = 5) × 1·8–3·1 (x = 2) µm.

Colony growth rate optimal at 25° on MEA, reaching a 44 mm diam. in 6 d. No growth occurred below 5° or above 35°. Able to withstand high concentrations of cycloheximide with a 40% reduction in growth on 2·5% cycloheximide after 6 d at 20° in the dark. Hyphae immersed in medium, mycelia hyaline to pale brown, smooth, frequently constricted at the septa, 2–8 ($\bar{x}$ = 4) µm diam. *Conidiophores* in groups of two or more, arising directly from the mycelium, erect, macronematous, mononematous, smooth, olivaceous to light brown, 117–229 ($\bar{x}$ = 169) µm long, rhizoids absent. Stipes olivaceous to light brown, smooth, cylindrical, simple, 1–6 septate, 40–170 ($\bar{x}$ = 102) µm long (from first basal septum to below primary branches), 4–9 ($\bar{x}$ = 6) µm wide below primary branches; apical and basal cells not swollen (Figs 1; 8*a*).



Figs 1–7. Light and scanning electron micrographs of the anamorph of *O. aenigmaticum*. **Fig. 1.** Light micrograph of a conidiophore (Bar, 10 µm). **Fig. 2.** Scanning electron micrograph of a conidiophore (bar, 10 µm). **Fig. 3.** Conidiogenous cells (bar, 1 µm). **Fig. 4.** Conidiogenous apparatus (bar, 10 µm). **Fig. 5.** Conidia (bar, 10 µm). **Fig. 6.** Perithecium (bar, 100 µm). **Fig. 7.** Ascospores (bar, 10 µm).

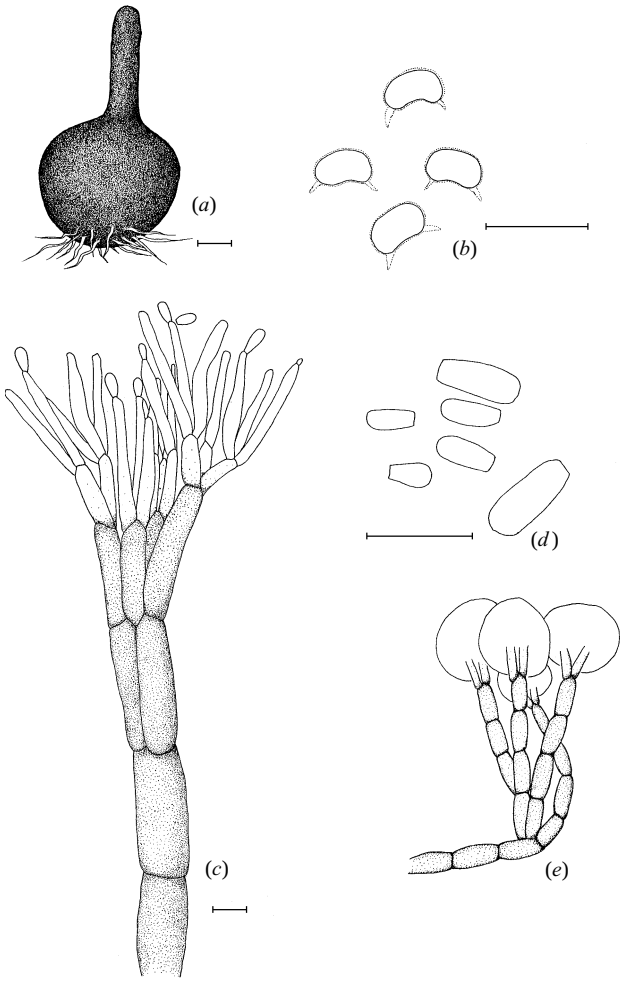


Fig. 8. *Ophiostoma aenigmaticum* with its *Leptographium* anamorph. (*a*) Perithecium (bar, 100 μm). (*b*) Ascospores surrounded with sheaths (bar, 10 μm). (*c*) *Leptographium* anamorph (bar, 10 μm). (*d*) Conidia (bar, 10 μm). (*e*) Schematic representation of a group of conidiophores with conidia accumulating in a mucilaginous mass.

Conidiogenous apparatus 34–85 ($\bar{x} = 64$) long, excluding the conidial masses, with two to four series of cylindrical branches; two primary branches, olivaceous, smooth, aseptate, 11–26 ($\bar{x} = 19$) μm long and 2–6 ($\bar{x} = 4$) μm wide, secondary branches hyaline to olivaceous, 0–1 septate, 11–23 ($\bar{x} = 17$) μm long, 2–5 ($\bar{x} = 3{\cdot}5$) μm wide; tertiary branches hyaline to olivaceous, aseptate, 9–22 ($\bar{x} = 15$) μm long, 2–4 ($\bar{x} = 3$) mm wide (Fig. 2). *Conidiogenous cells* discrete, 2–3 per branch, tapering slightly from the base to apex, cylindrical, straight, 10–23 ($\bar{x} = 17$) μm long and 1·8–2·5 ($\bar{x} = 2$) μm wide. Conidium development occurring through replacement wall building with holoblastic ontogeny and percurrent proliferation with delayed secession giving the false impression of sympodial proliferation (Minter *et al*., 1982, 1983; Van Wyk, Wingfield & Marasas, 1987) (Figs 3, 4). *Conidia* hyaline, aseptate, obovoid to allantoid, with rounded apices and truncate bases, 4–9 ($x = 5$) × 1·8–3·1 ($x = 2$) μm (Figs 5, 8*d*). Conidia accumulating in slimy droplets on top of the conidiogenous apparatus (Fig. 8*e*).

Specimens examined: Cultures on 2% malt extract agar, isolated from *P. jezoensis* infested with *I. typographus japonicus*, Hokkaido, Japan, June 1990, Y. Yamaoka, PREM 54870 (CBS 501.96). Holotype. Paratypes: isolated from *P. jezoensis* infested with *I. typographus japonicus*, Hokkaido, Japan, June 1990, Y. Yamaoka, PREEM 54871 (CBS 502.96), PREM 54872 (CBS 503.96), PREM 54873 (CBS 504.96).

Dried specimens are deposited in the herbarium of the Plant Protection Research Institute, Pretoria (PREM) and live isolates are deposited in the culture collection of the Centraalbureau voor Schimmelcultures (CBS).

DISCUSSION

In this study, employing many new collections of *O. huntii* and *O. europhioides*, we have been able to confirm their distinct nature, based on their morphology. Although *O. huntii* and *O. europhioides* have both been associated with pine-infesting insects and share a similar morphology, they can be distinguished from each other based on a number of characteristics. These include the abundant conidiophore production of *O. europhioides* and the sparsity of these structures in *O. huntii*. The typical serpentine growth pattern observed in *O. huntii* is also less obvious in *O. europhioides*.

Morphologically, while the taxa treated here (*O. huntii*, *O. europhioides* and *O. aenigmaticum*), are very similar, *O. europhioides* and *O. aenigmaticum* show the greatest resemblance. *O. aenigmaticum* can, however, be distinguished from the other two species based on its significantly smaller perithecia and elongated appendages of the ascospores. Both *O. europhioides* and *O. aenigmatum* were shown to be homothallic in this study, in contrast to *O. huntii*, which was found to be heterothallic. This also supports the findings of Zambino & Harrington (1992) who, using isozyme analyses, showed that *O. huntii* and *O. europhioides* clustered together in a group with *Leptographium lundbergii* Lagerb. & Melin.

Based on the distribution of the various isolates studied, these three taxa may prove to be distinguishable based on their ecological associations. *Ophiostoma huntii* has been known to occur mainly on pine and spruce trees and is associated with several different bark beetles, including *Dendroctonus ponderosae* Hopk., *Ips pini* Say, *Hylastes ater* Payk. and *Tomicus piniperda* (Robinson-Jeffrey & Grinchenko, 1964; Davidson & Robinson-Jeffrey, 1965; Harrington, 1988; Wingfield & Gibbs, 1991). *Ophiostoma europhioides* is also known to occur on pine and spruce, as well as douglas-fir, and has been associated with bark beetles in the genera *Dryocoetus* Eich., *Pityogenes* Bed. and *Ips typographus* L. (Davidson & Robinson-Jeffrey, 1965; Davidson *et al*., 1967; Solheim, 1986; Harrington, 1988; Yamaoka *et al*., 1997). In contrast, *O. aenigmaticum* has thus far been isolated only from spruce in Japan, where it is associated with the bark beetle *I. typographus japonicus* (Yamaoka *et al*., 1997). The association of both *O. europhioides* and *O. aenigmaticum* with *I. typographus* further suggests a close relationship between these two fungi.

Upadhyay's (1981) consideration of *O. europhioides* and *O. piceaperdum* as synonyms led us to question whether *O. aenigmaticum* could not perhaps be accommodated in *O. piceaperdum*. *O. piceaperdum* is also known to occur on spruce as well as pine (Rumbold, 1936; Hunt, 1956) and was described by Rumbold (1936) as having ellipsoid ascospores. Hunt (1956) noted that hat-shaped asocospores sometimes

occur in this fungus although we doubt the accuracy of this observation. Based on ascospore morphology, *O. aenigmaticum* can clearly not be accommodated in this species. Investigation of additional collections of *O. piceaperdum* and a study of the type material is necessary to define its morphological characters, and also its relationship with *O. europhioides*.

Ceratocystis pseudoeurophioides Olchow. & J. Reid, described by Olchowecki & Reid (1974), is a species similar to *O. europhioides*. These authors distinguished this species from *O. europhioides* based on the presence of a *Verticicladiella* S. Hughes anamorph in the former, in contrast to the *Leptographium* anamorph in the latter species. Upadhyay (1981) treated *O. pseudoeurophioides* as a synonym of *Ceratocystosis* (= *Ophiostoma*) *penicillata* (Grosmann) Siemaszko, which is very distinctive and it is unlikely that this observation is in doubt. We have not been able to locate the type material and have thus not been able to address this issue.

Ophiostoma huntii appears to be a relatively well-defined species associated with pine-root and stem infesting insects, both in North America and Europe (Robinson-Jeffrey & Grinchenko, 1964; Davidson & Robinson-Jeffrey, 1965; Wingfield & Gibbs, 1991). Isolates from Australia and New Zealand have evidently been introduced into these countries with the root-infesting bark beetle *Hylastes ater*, which is known to be associated with other ophiostomatoid species on pine in Europe (Wingfield & Gibbs, 1991). In this study, we showed that the fungus is heterothallic, and the fact that we were able to obtain fertile progeny from crosses between isolates from different continents leaves us with little doubt regarding its identity. This is the first record of the fungus from these countries and it might also suggest that it could have originated from a single introduction. A study of additional cultures would, however, be necessary to test this hypothesis.

Results of this study have added considerably to our knowledge of the distribution as well as to the taxonomy of two well-known conifer-associated fungi. This group of fungi is one that has significant quarantine importance and numerous species have already been introduced into new environments. An ability to identify species accurately is an essential component of any quarantine programme and studies such as this one should help to develop risk management strategies.

We would like to thank Mr L. van Rhyneveld for providing the Latin descriptions and the Foundation of Research Development for financial support. Various culture collections and colleagues noted in Table 1 are also thanked for their assistance in supplying material without which this study could not have been undertaken.

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(*Accepted 28 May 1997*)