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# Taxonomy of *Ophiostoma radiaticola* sp. nov. *(Ophiostomatales,* Ascomycetes), the teleomorph of *Pesotum pini,* isolated from logs of *Pinus radiata*

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**Abstract**—A new *Ophiostoma* species causing sapstain was isolated from *Pinus radiata* logs grown and stored in New Zealand, and imported from New Zealand to Korea. *Ophiostoma radiaticola* produces dark ascomata with long necks, lacks ostiolar hyphae and has hyaline reniform ascospores with a hat-shaped sheath. The fungus has mononematous *Leptographium*-like conidiophores that intergrade with synnematous *Pesotum*-like conidiophores, the latter previously described under the anamorph name *Pesotum pini* Mating tests on pine sapwood wafers demonstrated that *O. radiaticola* is a heterothallic species with two mating types. Phylogenetic analyses of aligned ITS 2/partial LSU rDNA, partial β-tubulin and partial actin DNA sequences demonstrate that *O. radiaticola* is a phylogenetically distinct species most closely related to *O. cainii* and *O. galeiformis*, with which it shares many morphological characters. It is also more closely related to *Ophiostoma* species with *Leptographium* anamorphs than to species of the *O. piceae* and *O. ulmi* complexes, the best-known groups of species with *Pesotum* anamorphs.

**Key word—**radiata pine, sapstain, *Pesotum; Ophiostoma cainii*, *Ophiostoma galeiformis*

## Introduction

Originating in a small area of coastal south western USA, radiata pine (*Pinus radiata* D. Don) is now the most important lumber species in New Zealand (1.33 million ha), Chile (1.36 million ha), Australia (720,000 ha) and Spain (260,000 ha) and is widely used in many Asian countries, particularly Korea and Japan (Price 1997). The tree and its wood

are susceptible to fungal attack, and there are active studies of sapstain fungi underway in countries growing or importing radiata pine. In New Zealand, surveys of sapstaining fungi that included radiata pine were undertaken by Hutchison & Reid (1988a, b), and Farrell et al. (1997), and additional species were reported in recent taxonomic literature (Jacobs & Wingfield 2001).

Many species of the ascomycete genus *Ophiostoma* cause blue-stain in logs and lumber, and those that have been reported from *P. radiata* in New Zealand are listed in Table 1. Other species cause diseases of living, standing trees (Boyce 1961, Wingfield & Gibbs 1991), or are associated with root diseases and vascular wilt diseases such as Dutch Elm Disease (Harrington 1988, 1993, Wingfield et al. 1988, Wingfield et al. 1993). Many *Ophiostoma* species are vectored by bark beetles (Coleoptera: Scolytidae) that infest species of *Pinus* and other conifers (Harrington 1988, 1993, Wingfield et al. 1993). *Ophiostoma* presently includes slightly more than 100 species, characterized by the production of superficial, generally darkly pigmented, thin-walled ascomata with evanescent asci produced in a dark globose base, with variously-shaped ascospores in most species exuding from a long, ostiolate neck. The genus is well-known for its diversity of anamorphs, and the taxonomic controversies that have surrounded both the teleomorph and the anamorphs (Wingfield et al. 1993). Presently, mycelial anamorphs are classified in *Sporothrix* Hekt. & Perk. and *Hyalorhinocladiella* Upadh. & Kendr., macronematous anamorphs are classified in *Leptographium* Lagerb. & Melin, and synnematous anamorphs are classified in *Pesotum* Crane & Schokn. (and sometimes also in *Phialographium* Upadh. & Kendr.).

Molecular phylogenetic studies combined with the application of biological species concepts derived from *in vitro* mating tests have begun to reveal the infrageneric structure of *Ophiostoma* and are resulting in more robust species concepts. In general, there is good correlation between monophyletic clades based on phylogenetic analysis of rDNA alignments and groups distinguished by combinations of ascomatal, ascospore and anamorph characters. The structure and species composition of two fairly large clades within the genus are now relatively clear. The *Piliferum* clade includes species mostly with dark, long-necked ascomata with ostiolar hyphae, with orange-section shaped ascospores lacking a sheath, with *Sporothrix* anamorphs and in many of the species also a synnematous *Pesotum* anamorph. This clade includes the type species of *Ophiostoma*, *O. piliferum*, as well as several other species complexes. These comprise complexes of reproductively isolated, sometime geographically isolated, sibling species that are often distinguished by only inconspicuous morphological characters. Included are the *O. piliferum* complex (Schroeder et al. 2001), the Dutch Elm Disease complex (Brasier 1993, Brasier & Mehrotra 1995), the *O. piceae* complex (Brasier 1993, Harrington et al. 2001), and the *O. minus* complex (Gorton & Webber 2000, Jacobs & Kirisits 2003). A second major clade, the *Leptographium* clade, includes *Ophiostoma* species with macronematous *Leptographium* anamorphs, and several apparently strictly anamorphic species (Jacobs et al. 2001). To date, less work has been done on elucidating biological species in this clade and it is likely that complexes of sibling species in this group remain to be discovered. Our study focuses on some species in this second major clade.

Recently, we isolated an unidentified *Ophiostoma* species from radiata pine logs in New Zealand and from logs imported from New Zealand into Korea. This species was distinctive in having an anamorph with a continuum of morphologies between *Leptographium* and *Pesotum*. We compared the morphological and molecular features of this unidentified species with *O. cainii* (Olchow. & J. Reid) T.C. Harr. and *O. galeiformis* (B.K. Bakshi) Math.-Käärik, two species with similar ascospores and a similar combination of anamorphs, and the anamorphic species *Pesotum pini* (Hutchison & Reid) Okada & Seifert. The fungus is described here as a new heterothallic species, *O. radiaticola*, the teleomorph of *P. pini*.

**Table 1.** *Ophiostoma* species and related anamorphs reported on *Pinus radiata* in New Zealand in previous studies

| Species | Reference |
|---|---|
| *L. euphyes* Jacobs & Wingf. | Jacobs & Wingfield 2001 |
| *L. lundbergii* Lagerb. & Melin | Jacobs & Wingfield 2001 |
| *L. procerum* (Kendr.) Wingf. | Farrell et al. 1997, Jacobs & Wingfield 2001 |
| *L. truncatum* (Wingf. & Mararas) Wingf. | Farrell et al. 1997, Jacobs & Wingfield 2001 |
| *Ceratocystis coronata* Olchow. & Reid | Hutchison & Reid 1988a, Farrell et al. 1997 |
| *O. flocossum* Math.-Käärik | Farrell et al. 1997 |
| *O. huntii* (Rob.-Jeff.) Hoog & Scheffer | Farrell et al. 1997, Jacobs & Wingfield 2001 |
| *O. ips* (Rumbold) Nannf. | Hutchison & Reid 1988a, Farrell et al. 1997 |
| *O. piceae* (Münch) H. & P. Sydow | Hutchison & Reid 1988a, Farrell et al. 1997 |
| *O. piceaperdum* (Rumbold) Arx | Hutchison & Reid 1988a, Jacobs & Wingfield 2001 |
| *O. piliferum* (Fr.) H. & P. Sydow | Hutchison & Reid 1988a, Farrell et al. 1997 |
| *O. pluriannulatum* (Hedg.) H. & P. Sydow | Farrell et al. 1997 |
| *O. quercus* (Georg.) Nannf. | Farrell et al. 1997 |
| *O. setosum* Uzunovic et al.[1] | Farrell et al. 1997 |
| *O. stenoceras* (Robak) Melin & Nannf. | Farrell et al. 1997 |

[1] Reported as "*Graphium* sp. with black-veined synnema"

## Materials and methods

### Morphological and cultural studies

Cultures were isolated from stained radiata pine logs during the winters of 1998–2000 in Korea by Kim & Kim (2000) and Kim (2001). Four strains (NZFS 559, 613, 1080, 3534, and 5190) originating from radiata pine in New Zealand were obtained from Mrs. M. Dick (NZFS, Forest Research Culture Collection, Rotorua, New Zealand). Representative cultures are maintained in the Culture Collections of Korea University (KUC, Seoul, Korea), the Breuil Culture Collection (BUBC, University of British Columbia, Vancouver, BC, Canada), and in the Canadian Collection of Fungal Cultures (DAOM, Ottawa, ON, Canada).

For morphological studies, cultures were grown on 2% Malt Extract Agar (D-MEA, 20 g Difco malt extract, 15 g Difco agar, and 1000 mL distilled water). Growth rates were determined at 5 C intervals between 5 and 35 °C. Agar disks (5 mm in diameter) taken from the edge of a freshly grown colony were placed on 2% D-MEA media in 90 mm Petri dishes with three replicates per isolate. Colony diameter on each plate was measured 3, 5, and 7 days after inoculation, respectively. Growth rates were calculated in mm per day. Cycloheximide tolerance of isolates was examined by measuring their growth at 25 °C on D-MEA amended with different concentrations (0.05, 0.1, and 0.5%) of cycloheximide (Aldrich chem. Co., WI, USA).

Morphological observations were made on fungal structures produced on 2% D-MEA and sterile lodgepole pine sapwood wafers. For light microscopy, fungal structures were mounted in water or 90% lactic acid and observed using a Zeiss Axioplan or an Olympus BX50 light microscope. Means ± standard error (se) statistics are based on 25 measurements per structure. For scanning electron microscopy (SEM), small wood blocks (10 x 2 x 7 mm) covered with conidiophores were fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate at pH 7.1. All fixation procedures were performed within 1 hr under vacuum. The samples sat for 1 min, were microwaved for 40 s at 212 watts, and held for 3 min. Then the samples were rinsed with buffer (0.05 M cacodylate, pH 7.1) for 5 min, microwaved for 40 s, and then fixed with 2% osmium tetroxide. After a brief rinse with sterilized water, the samples were dehydrated in a grade ethanol series (50, 70, 85, 95, 100, and 100%). For each ethanol series, the samples sat for 1 min and were then microwaved for 40 s at 115 watts. After fixation, the samples were dried with a Balzers CPD 020 critical point drier, coated with a Nanotech Semprep II sputter coater, and examined using an Hitachi S4700 scanning electron microscope.

### Mating experiments

Sterile lodgepole pine (*Pinus contorta* var. *latifolia* Engelm.) sapwood wafers were used for mating tests, following the method of Brasier (1981). The wood was placed directly on 1% agar media to maintain moisture content. Three types of mating experiments were undertaken. All single spore isolates of *O. radiaticola* were crossed, single spore

isolates of *O. galeiformis* were crossed, and strains of the two species were crossed with each other in all possible combinations and then incubated, for 4-5 weeks at room temperature. Single ascospore isolates subsequently obtained from the progeny of such crosses were backcrossed to the parental mating strains to confirm the fertility of the F1 generation. In addition, single strains of each species were inoculated onto test blocks to test for homothallic ascomatal formation.

### DNA extraction, PCR, sequencing and phylogenetic analysis

For genomic DNA preparation, cultures were inoculated on 2% Oxoid MEA (O-MEA) overlaid with sterile cellophane sheets (Bio-Rad) and grown for 7 days at room temperature. DNA extraction was carried out using the methods described by Kim et al. (1999).

The internal transcribed spacer (ITS) 2 and partial large subunit (LSU) regions of the ribosomal DNA operon (rDNA) were amplified using the primers ITS3 and LR3 (Vilgalys & Hester 1990, White et al. 1990). Amplification of part of the actin gene was done with the primers Lepact F and Lepact R (Lim et al. 2004). A part of the β-tubulin gene was amplified using the primers Bt2E (5'-GTT CAY CTC CAG ACC GCY CAG TG-3') and BT12 (Kim et al. 2003). PCR amplification was performed as described by Lee et al. (2003). PCR products were visualized by electrophoresis on a 1.4 % agarose gel containing ethidium bromide. The PCR products were purified using a Qiaquick PCR Purification Kit (Qiagen, Mississauga, Ontario). Purified PCR products were sequenced using the same primer sets described above. Sequencing was performed on an ABI 3700 automated sequencer (Perkin-Elmer Inc. Foster City, California) at the DNA synthesis and Sequencing Facility, MACROGEN (Seoul, Korea). Sequences were proofread and edited using the PHYDIT program version 3.2 (http://plasza.snu.ac.kr/~jchun/phydit/). The new sequences derived in this study are indicated by bold type in Figs 16 and 17. Three different data sets were assembled and aligned including other ophiostomatoid fungi retrieved from GenBank (accession numbers in normal type in Figs. 16, 17).

For phylogenetic analysis, the three data sets representing the three gene regions sequenced were aligned using Clustal X program (Thompson et al. 1997) and then optimized using the PHYDIT program version 3.2. Ambiguous regions of the rDNA alignment and intron regions of actin and β-tubulin genes were excluded from analyses. Based on previous phylogenetic analyses (Ayliffe et al. 2001, Jacobs et al. 2001, Spatafora & Blackwell 1994), the phylogenetic trees of the rDNA, the actin and the β-tubulin genes were rooted with *Ophiostoma ips* (AY194934), *O. ulmi* (Buisman) Nannf. (AF378563), and *O. ips* (AY194950), respectively. All parsimony analyses used the heuristic search option with simple addition sequences with TBR branch swapping and MULPARS turned on. Gaps were treated as missing data. Branch stability was assessed by 1000 replicate bootstrap replications using heuristic searches as implemented in PAUP*4.0b10 (Swofford 2001).

## Results

### Taxonomic description

***Ophiostoma radiaticola*** J.-J. Kim, Seifert & G.-H. Kim, **sp. nov.** Figs. 1–14.

Anamorph: *Pesotum pini* (Hutchison & Reid) Okada & Seifert, Can. J. Bot. 76: 1504. 1998.

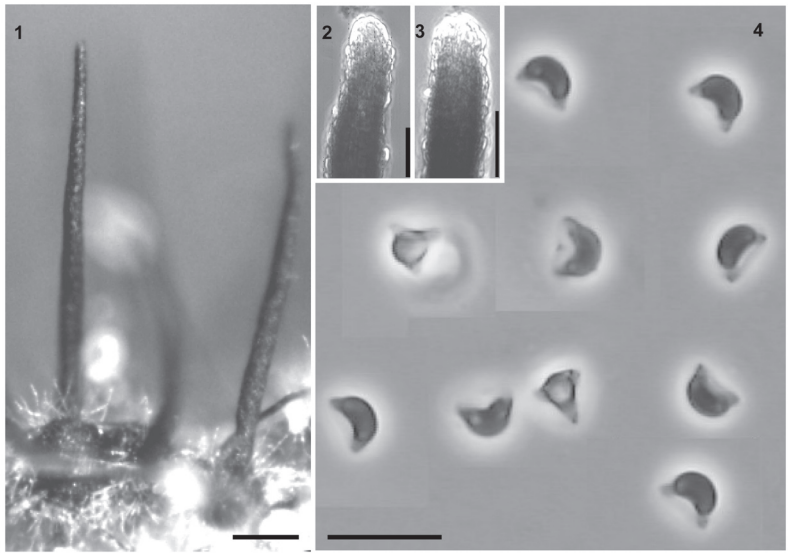
≡ *Hyalopesotum pini* Hutchison & Reid, N. Z. J. Bot. 26: 90. 1988.

*Species similis Ophiostomatis cainii et Ophiostomatis galeiformis sed genetice isolata, heterothallica. Ascomata atra, e basi globosa, 200–300 µm diam., et collo 300–960 µm alto composite; collum ad basim 40–50 µm, in crista hypharum ostiolarium absente. Asci non visi. Massa ascosporarum apicalis mucida vel in cirrhum, alba. Ascosporae hyalinae, reniformes vel allantoideae, 4–4.5 × 1.5–3 µm, vagina gelatinosa circumdatae, vagina a latere visa cucullata et e polo triangulais. Conidiomata synnematica typica generis Pesoti, basi fusca vel atra, 400–680 µm alta, 15–150 µm lata. Conidiophora penicillata, biverticillata vel terverticillata. Cellulae conidiogenae cylindricae vel subulatae, 6.5–20.5 × 1.5–2 µm, phialidicer vel percurrenter vel sympodialiter proliferentes. Massa conidiorum apicalis mucida, alba, flava vel armeniaca. Conidia 3–6.5 × 1.5–2 µm, oblongo-ellipsoidea vel plusminusve clavata. Conidiophora et conidia typica generis Leptographii similis conidiophora in synnematicis. Culturae in extracto malti 'Difco' 25 C in dies circa 2.7 mm radii crescentes.* ***Holotypus*** *in ligno Pini contorae, combinatio culturarum purarum compatibilium KUC2036 et NZFS1080, herb. DAOM no. 234021.*

***Etymology***: *radiaticola,* indicating growth on radiata pine.

Ascomata developing about 4 weeks after pairing of two sexually compatible strains on sterilized lodgepole pine wafers, not produced on agar; sparse, solitary, superficial, intermingled with the anamorph; the base dark brown to black, smooth, globose, collapsing cupulate when dry, 200–300 µm diam (mean ± se = 255 ± 30), with pale brown hyphae making the surface appear somewhat hairy, the surface composed more or less textura angularis of brown cells up to 3–15 µm in their longest dimension, the walls irregularly thickened up to 1.5 µm thick, with irregular deposits of darker pigment; the neck black, light brown near the apex, smooth under the dissecting scope but somewhat scurfy under higher magnification, curved or straight, subulate, 300–960 µm long (mean ± se = 740 ±194), 40–50 µm wide (mean ± se = 49 ± 3.2) at the base, 10–15 µm wide at the tip; composed of more or less parallel hyphae, individually brown, opaque in mass, 2–3.5 µm wide, frequently septate, constricted at the septa; becoming paler abruptly at the tip of the neck; ostiolar hyphae absent. Asci not observed. Ascospore masses extrude in a thick, whitish mass, globose to cirrhus-like. Ascospores 4–4.5 × 1.5–3.0 (mean ± se = 4.5 ± 0.4 × 2.8 ± 0.8) µm, reniform in side view often with one strongly concave surface, appearing hat-shaped because of triangular sheath extensions at the ends, ellipsoidal in face view, round or ellipsoidal in end view but appearing triangular because of the sheath, sheath up to about 1–1.5 µm thick.

Synnemata solitary or gregarious, 400–680 µm tall (mean ± se = 535 ± 86), 15–150 µm wide at the base, the stipe cylindrical or subulate, pale to dark brown or black at the base, reddish brown towards the apex. Hyphae of stipe 2.5–4 µm wide at the base, brown to olivaceous, with walls 0.5–1 µm thick, and 2–3 µm below the capitulum, olivaceous,




**Figs. 1–4**. *Ophiostoma radiaticola*, teleomorph, from the holotype. 1. Ascomata on wood blocks. 2, 3. Ostiolar regions of two ascomata, showing absence of ostiolar hyphae. 4. Ascospores showing hat-shape in side view, and triangular shape of the ascospores in end view. Scale bars: Fig. 1 = 200 $\mu$m; Figs. 2, 3 = 20 $\mu$m; Fig. 4 = 10 $\mu$m.

with the walls slightly thickened, and thin septa present. Conidiophore branching biverticillate to terverticillate, lower whorls often with 2 branches, conidiogenous apparatus 42.5–80.5 (mean ± se = 61.2 ± 13.4) µm long, basal branches 9.5–31.5 (mean ± se = 19.1 ± 5.7) × 1.5–2.5 µm, cylindrical, brown, secondary branches 8.0–16.0 (mean ± se = 12.5 ± 2.7) × 2.5–3 µm, metulae hyaline, 9.5–14.0 (mean ± se = 11.3 ± 1.6) × 1–3 µm. Conidiogenous cells proliferating sympodially or percurrently, in whorls of 2–3, 6.5–20.5 (12.4 ± 3.8) × 1.5–2 µm, more or less cylindrical or subulate, sometimes uneven in outline, the walls usually slightly thickened at the base, conidiogenous aperture 1–1.5 µm wide, apparent periclinal thickening and slightly flared collarette visible only with phase contrast. Conidial mass white, light yellow or light orange when dry, pale brown when dry, globose, turbinate, ovate or ellipsoidal, 75–125 µm diam. Conidia aseptate, varying greatly in size but more or less uniform in shape, 3.0–6.5 × 1.5–2.0 (mean ± se = 4.7 ± 0.9 ×1.6 ± 0.2) µm, oblong-ellipsoidal to cylindrical, sometimes clavate, L/B 1.8–2.8, sometimes slightly curved, base often somewhat truncate.

Mononematous anamorph *Leptographium*-like, solitary or in all intermediate forms to complete synnemata, or emerging as a terminal extension to a short synnema. When mononematous, usually with a tightly appressed, longer conidiogenous apparatus 200–500 (x mean ± se = 325 ± 97) µm; stipe straight or flexuous, pale to dark brown, 5–10 septate, 3.0–5.0 (mean ± se = 4.3 ± 0.6) µm wide at the base. Conidia identical to those in synnemata.

The optimal growth temperature for *O. radiaticola* was 25 °C with a growth rate of 2.7 mm/day on 2% D-MEA. No growth was observed below 10 °C or above 35 °C. On cycloheximide (0.05, 0.1, and 0.5%)-amended MEA, the mean growth rates of three isolates of *O. radiaticola* were 2.2, 2.0, and 1.4 mm per day, respectively.Colonies were effuse, spreading, first white, becoming olivaceous brown (4E8) on D-MEA. Hyphae mostly immersed, hyaline to subhyaline, smooth, 1.5–4.5 µm thick.

**SPECIMENS AND CULTURES EXAMINED: HOLOTYPE**: Mating between the cultures KUC 2036 and NZFS 1080, listed below (DAOM 234021). KOREA. INCHEON: *Pinus radiata* logs imported from New Zealand, March 2000, J.-J. Kim and G.-H. Kim, KUC 2036 (DAOM 233237); *Pinus radiata* logs imported from New Zealand, March 2000, J.-J. Kim and G.-H. Kim, KUC 2037. NEW ZEALAND: *Pinus radiata* 1994, M. Dick, NZFS 3534; *Pinus radiata* 1997, M. Dick, NZFS 5190; *Pinus radiata* 2001, M. Dick, NZFS 559, NZFS 613 (DAOM 233239). *Pinus radiata*, M. Dick, NZFS 1080. HOLOTYPE of *Hyalopesotum pini*, on *Pinus radiata*, J. Reid no. 88, New Zealand, near Onemana, Tairua State Forest, Coromandel, 20 May 1982 [WIN(M)].

## Mating experiments

Isolates of *O. radiaticola* segregated clearly into two mating types. The ascomata from these crosses were fertile and single ascospore progeny were easily obtained. Randomly selected single ascospore progeny, when mated against either parent, also produced viable ascomata. *O. radiaticola* is heterothallic, and single ascospore isolates do not produce ascomata. Of the isolates used in these experiments, *O. radiaticola* KUC2036 and NZFS613 and the ex-type culture of *Pesotum pini* (UAMH9691) were of the (+) mating type and the others were (-) mating type (Table 2).

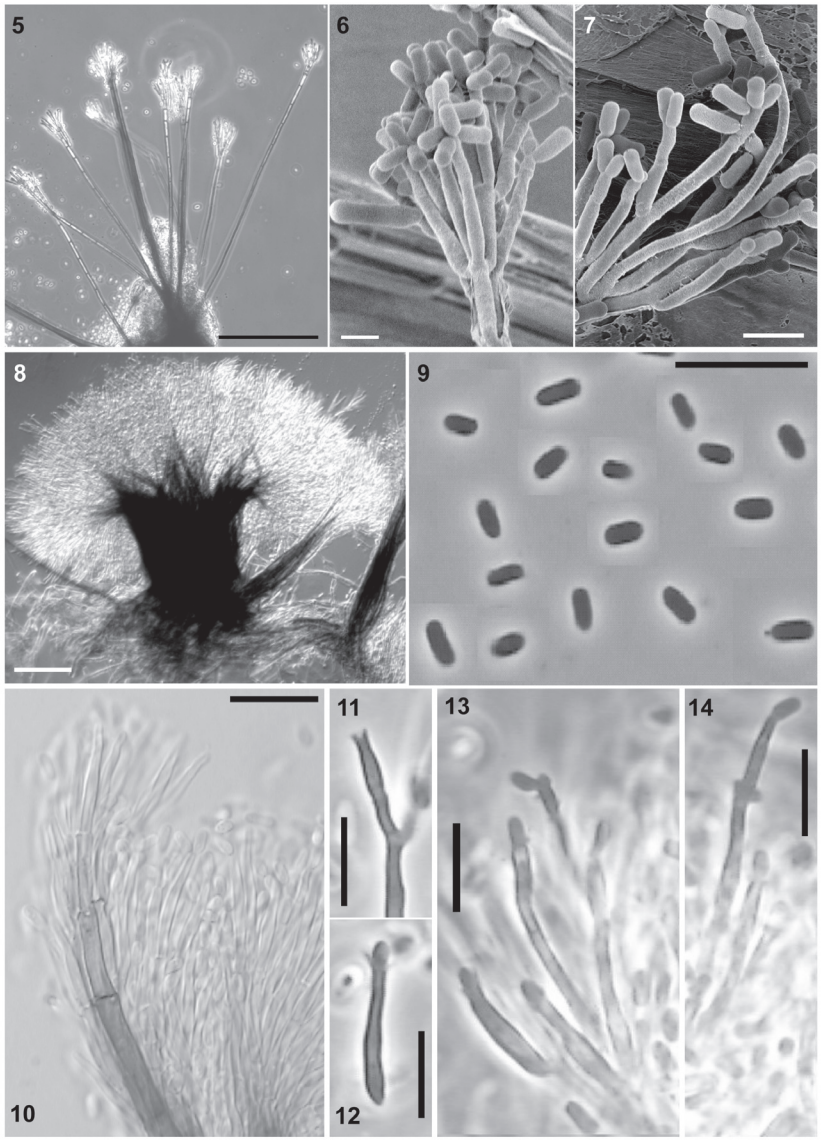
**Table 2**. Mating experiments of *Ophiostoma radiaticola*.[a]

| | Recipient strain | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Donor strain | **A** | **B** | **C** | D | E | F | G | H |
| **KUC2036 (A)** | –[b] | – | – | + | + | + | + | + |
| **NZFS613 (B)** | – | – | – | + | + | + | + | + |
| **UAMH9691 (C)** | – | – | – | + | + | + | + | + |
| KUC2037 (D) | + | + | + | – | – | – | – | – |
| NZFS1080 (E) | + | + | + | – | – | – | – | – |
| NZFS559 (F) | + | + | + | – | – | – | – | – |
| NZFS5190 (G) | + | + | + | – | – | – | – | – |
| NZFS3534 (H) | + | + | + | – | – | – | – | – |

[a] The strains in bold are (+) mating type and the others are (-) mating type.

[b] + indicates formation of ascomata with viable ascospores; – indicates no ascomata.

In our experiments, the three strains of *O. galeiformis*, CMW5290 (ex-epitype), CMW4447, and CMW4426 appeared to be homothallic. There was no evidence of mating between *O. radiaticola* and *O. galeiformis*, although this was difficult to interpret with certainty because of the homothallism of the latter species.




**Figs. 5–14**. *Ophiostoma radiaticola*, anamorphs, from holotype and ex-type culture. 5. *Leptographium*-like conidiophores from D-MEA. 6, 7. SEM micrographs of *Leptographium*-like conidiophores, showing percurrent and false-sympodial proliferation of conidiogenous cells. 8. *Pesotum*-like synnemata from D-MEA culture. 9. Conidia. 10. Conidiophores from synnemata, with DIC. 11-14. Conidiogenous cells from synnemata, with phase contrast, showing collarette-like flaring apparently representing phialidic conidiogenous cells. Scale bars: Fig. 5 = 100 $\mu$m; Figs. 6, 7 = 5 $\mu$m; Fig. 8 = 50 $\mu$m; Figs. 9-14 = 10$\mu$m.

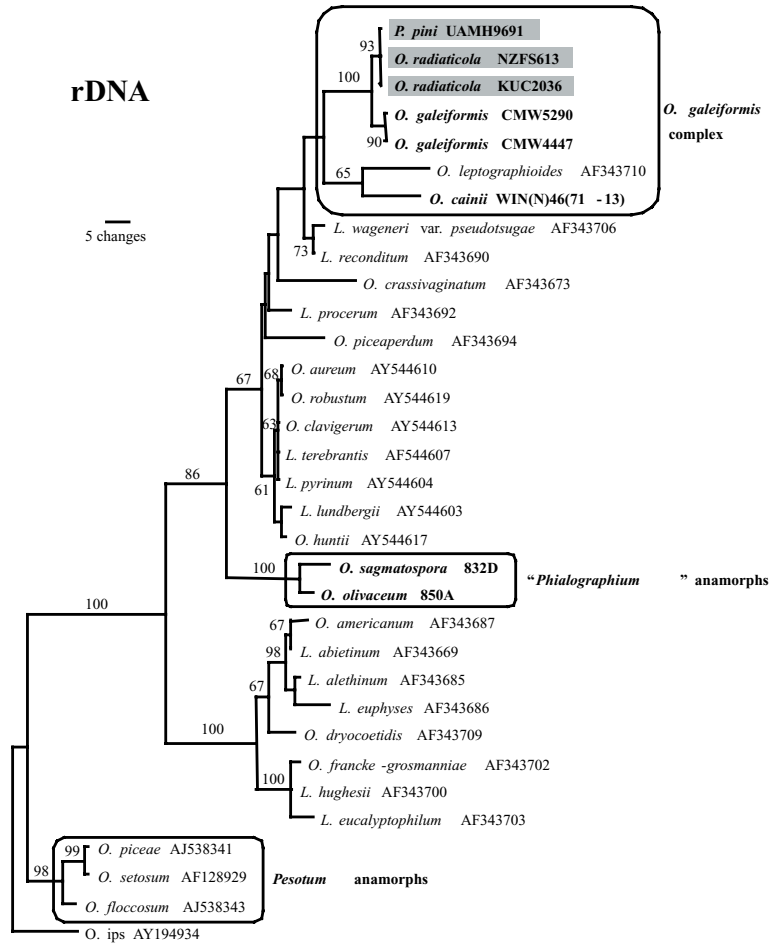



**Fig. 15**. Phylogenetic tree based on rDNA sequences, showing the relationships among *O. radiaticola* and its anamorph *P. pini*, *O. galeiformis* and *O. cainii*, and the position of this clade among other *Ophiostoma* species. Species with *Pesotum* or "*Phialographium*" anamorphs are indicated; other species have *Leptographium* anamorphs. Numbers on selected branches represent bootstrap values above 60%. The tree had the highest log-likelihood value among 62 equally parsimonious trees. The three branches with arrows would collapse in the strict consensus tree based on the MPTs. GenBank accession numbers obtained in this study are as follows: *P. pini* UAMH9691 (AY744549); *O. radiaticola* NZFS613 (AY744550), KUC2036 (AY744551); *O. galeiformis* CMW5290 (AY744552), CMW4447 (AY744553); *O. cainii* WIN(N)46(71-13) (AY744548); *O. sagmatospora* 832D (AY744546); and *O. olivaceum* 850A (AY744547).

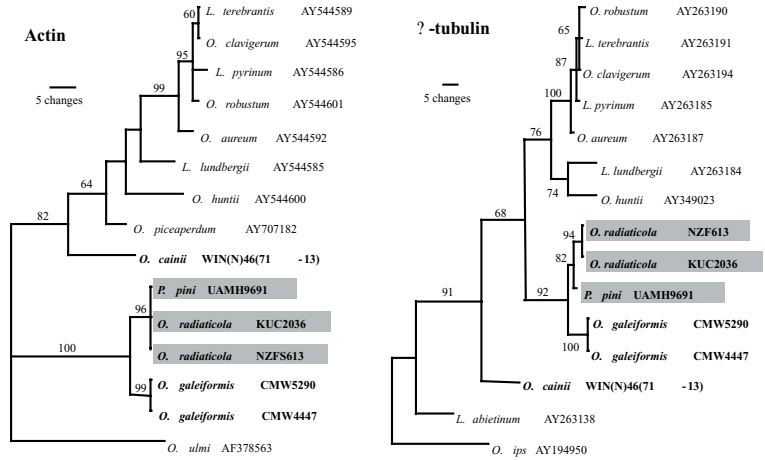



**Fig. 16**. Actin and β-tubulin gene trees of a subset of taxa including the *O. galeiformis* complex, confirming the conspecificity of *O. radiaticola* and its anamorph *P. pini*, and its sister species relationship with *O. galeiformis*. GenBank accession numbers for actin[a] and β-tubulin[b] gene obtained in this study are as follows: *P. pini* UAMH9691 (AY744554[a], AY744560[b]); *O. radiaticola* KUC2036 (AY744555[a], AY744562[b]), NZFS613 (AY744556[a], AY744561[b]); *O. galeiformis* CMW5290 (AY744557[a], AY744563[b]), CMW4447 (AY744558[a], AY744564[b]); and *O. cainii* WIN(N)46(71-13) (AY744559[a], AY744565[b]).

## Sequence alignments and phylogenetic analyses

The alignment of rDNA sequences containing ITS 2 region and 5' partial LSU region included 33 sequences representing 30 taxa (Fig. 15). After the introduction of gaps, the alignment included 658 nucleotide positions. Areas of ambiguous alignment were excluded from the analysis, and of the remaining 592 characters, 434 characters were constant, 44 were parsimony-uninformative, and 114 characters were parsimony-informative. Parsimony analysis of the rDNA sequences yielded 62 most parsimonious trees (MPT), for which the tree length (TL) was 303 steps, the consistency index (CI) was 0.7096, and the retention index (RI) was 0.8726. The tree shown in Figure 16 had the highest log-likelihood value (-lnL = 2602.15736) of the 62 MPTs.

Phylogenetic analyses using the rDNA data set demonstrated that some species with *Phialographium* anamorphs were related to the *Leptographium* clade, and suggested that *O. radiaticola*, with its mixed phialidic and sympodial synematous anamorphs, was a distinct species, closely related to *O. galeiformis* (Fig. 15). The three rDNA sequences of the *O. radiaticola* were identical and 98.9% similar to the two identical

sequences of *O. galeiformis*. These species were also closely related to *O. cainii* and *O. leptographioides* (R.W. Davidson) Arx. Because of the smaller number of sequences in GenBank, parsimony analyses of actin and β-tubulin genes sequences were undertaken for a subset of taxa closely related to *O. radiaticola*. Following the exclusion of intron regions, the alignment of the partial actin/β-tubulin genes included 677/550 nucleotide characters, of which 574/440 were constant, 42/38 were parsimony-uninformative, and 60/72 characters were parsimony-informative. Parsimony analyses of the partial actin/β-tubulin genes both yielded a single MPT (TL=148/184, CI=0.8041/0.7283, RI=0.8802/0.7917). MPTs for each of the actin and β-tubulin gene data sets are shown in Figure 16a and 16b, respectively. In these trees, the three isolates representing the new species constituted a distinct clade with highly supported bootstrap values alongside *O. galeiformis*.

The three molecular data sets were completely congruent concerning the relationships among *O. radiaticola, O. galeiformis*, *O. cainii* and *Pesotum pini*. *Ophiostoma radiaticola* was a sister group to *O. galeiformis* in all analyses, with the ex-type culture of *P. pini* grouping with the former species in all analyses. The position of the morphologically similar *O. cainii* was less clear, forming a poorly supported sister group along with *O. leptographioides* to *O. radiaticola* and *O. galeiformis* in the rDNA tree, placed basal to this clade in the β-tubulin tree, and in a separate clade in the actin tree.

## Discussion

*Ophiostoma radiaticola* is characterized by the production of slightly hairy, black ascomata with long black necks, lacking ostiolar hyphae, and with reniform ascospores surrounded by a sheath. It produces a gradation between mononematous *Leptographium*-like anamorphs and synnematous *Pesotum*-like anamorphs, producing identical conidia and conidiogenous cells. The conidiophores are unusual for having a mixture of phialidic (Figs. 11-14) and sympodially proliferating (Figs. 6, 7) conidiogenous cells. Hutchison and Reid (1988b) commented on this apparent mixture of conidiogenous types in *Pesotum pini*, suggesting that younger conidiogenous cells were phialidic, and became more typically sympodial as the conidiogenous locus proliferated. Based on our mating experiments, *O. radiaticola* meets the criteria as a distinct biological species, and the concordance among the three gene trees meet the criteria of the phylogenetic species concept (Taylor et al. 2000).

*Ophiostoma radiaticola* belongs to a group of three morphologically similar species with *O. cainii* and *O. galeiformis*, and we have provided a key to these three species below. Zhou et al. (2004) referred to this as the *O. galeiformis* complex, which we believe is appropriate because this is the oldest species name in the group. Each species has ascomatal necks lacking ostiolar hyphae, similar kidney shaped ascospores surrounded by a sheath that makes them seem hat-shaped in side view and triangular or quadrangular in end view, and macronematous anamorphs with oblong-ellispoidal, truncate conidia that vary considerably in size. In cultures of all three species, the anamorph is a mixture of mononematous, *Leptographium*-like conidiophores and synnematous conidiomata

that could be ascribed to *Pesotum*. In *O. cainii* and *O. radiaticola*, the anamorph in nature is mostly synnematous; details of the *in vivo* anamorph of *O. galeiformis* have yet to be recorded. This tendency to produce a continuum of anamorphs that could be ascribed to *Leptographium* in the one extreme, and *Pesotum* on the other extreme, has led to some confusion about how to most appropriately name the anamorph of *O. galeiformis* (Wingfield 1993). In fact, the species was not included in the monograph of *Leptographium* by Jacobs and Wingfield (2001), nor in the monograph of *Ceratocystis* and *Ophiostoma* by Upadhyay (1981). Zhou et al. (2004) recently presented a reconsideration of *O. galeiformis*, documenting its global distribution and providing a new description based on freshly isolated cultures. They considered the species heterothallic, although some strains (include the newly designated ex-epitype) appeared to be homothallic. The three strains of *O. galeiformis* that we received from them produced fertile ascomata on wood blocks without mating and thus appear to be homothallic. It is possible that our *O. radiaticola* is identical with the "*O. galeiformis*-like" fungus mentioned by Zhou et al. (2004), which was isolated in Mexico and the USA from three different pine species, including *P. radiata*.

Phylogenetically, *O. radiaticola, O. galeiformis* and *O. cainii* are related to other species with *Leptographium* anamorphs, rather than other species with *Pesotum* anamorphs. The *Leptographium* clade also includes some of the synnematous anamorphs of *Ophiostoma* species that have phialidic conidiogenous cells, which have been ascribed by some authors to *Phialographium*. Harrington et al. (2001) favoured the retention of *Phialographium* as distinct from *Pesotum* (typified by the anamorph of *O. ulmi*, in the Piliferum clade) for phylogenetic reasons. However, the phylogenetic relationship of *Leptographium* and *Phialographium* species evident in our phylogenies, combined with the tendencies of *O. radiaticola* and *O. cainii* to produce a mixture of conidiogenous cell types, leaves the question of whether acceptance of *Phialographium* makes things simpler or more complex. The anamorphs of *O. radiaticola, O. cainii* and *O. galeiformis* could be ascribed to any one of three genera, *Leptographium, Pesotum* or *Phialographium*. We have chosen to leave the anamorphic epithet of our new species in *Pesotum,* suggesting that the holomorph name should be used when discussing these fungi, in line with the recommendation of the International Code of Botanical Nomenclature that discourages the routine proposal of anamorph species names when unambiguous teleomorph names are available (Art. 59, rec. A3, Greuter et al. 2000).

### Key to the sibling species of the *O. galeiformis* complex

1. Ascospores mostly less than 4 × 2 um (without sheath) . . . . . .*O. galeiformis*
1. Ascospores mostly larger than 4 × 2 um (without sheath) . . . . . 2

2. Ascospore sheaths with four points in end view, synnemata fan-shaped in culture, reddish brown, known only from North America . . . . . *O. cainii*
2. Ascospore sheaths triangular in end view, synnemata with distinct stalk in culture, brown to blackish stipe, known from New Zealand and Korea . . . *O. radiaticola*

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## References

Ayliffe MA, Dodds PN, Lawrence GJ. 2001. Characterisation of a β-tubulin gene from *Melampsora lini* and comparison of fungal β-tubulin genes. Mycological Research 105: 818–826.

Boyce JS. 1961. Forest pathology. 3rd ed. McGraw-Hill Book Company, New York. 572 pp.

Brasier CM. 1981. Laboratory investigation of *Ceratocystis ulmi*. *In* Compendium of Elm Diseases (eds. R. J. Stipes & R. J. Campana), pp. 76–79. American Phytopathological Society Press: St Paul, Minnesota.

Brasier CM. 1993. The genetic system as a fungal taxonomic tool: Gene flow, molecular variation and sibling species in the '*Ophiostoma piceae-Ophiostoma ulmi*' complex and its taxonomic and ecological significance. *In Ceratocystis* and *Ophiostoma* taxonomy, ecology, and pathogenicity (eds. M. J. Wingfield, K. A. Seifert & J. F. Webber), pp. 77–92. The American Phytopathological Society Press: St. Paul, Minnesota.

Brasier CM, Mehrotra MD. 1995. *Ophiostoma himal-ulmi* sp. nov., a nw species of Dutch Elm disease fungus endemic to the Himalayas. Mycological Research 99: 205–209.

Farrell RL, Duncan SM, Ram AP, Kay SJ, Hadar E, Hadar Y, Blanchette RA, Harrington TC, McNew D. 1997. Causes of sapstain in New Zealand. *In* Strategies for Improving Protection of Logs and Lumber (ed. B. Kreber), pp. 25–29. New Zealand Forest Research Institute Bulletin No. 204.

Gorton C, Webber JF. 2000. Reevaluation of the status of the bluestain fungus and bark beetle associate *Ophiostoma minus*. Mycologia 92: 1071–1079.

Greuter W, McNeill J, Barrie FR, Burdet HM, Demoulin V, Filgueiras TS, Nicolson DH, Silva PC, Skog JE, Trehane P, Turland NJ, Hawksworth DL (eds.). 2000. International Code of Botanical Nomenclature (Saint Louis Code) adopted by the Sixteenth International Botanical Congress St. Louis, Missouri, July - August 1999.Koeltz Scientific Books, Königstein.

Harrington TC. 1988. *Leptographium* species, their distributions, hosts and insect vectors. *In Leptographium* root diseases on conifers (eds. T. C. Harrington & F. W. Cobb, Jr.), pp. 1–39. The American Phytopathological Society. St. Paul, Minnesota.

Harrington TC. 1993. Diseases of conifers caused by species of *Ophiostoma* and *Leptographium*. *In Ceratocystis* and *Ophiostoma* taxonomy, ecology, and pathogenicity (eds. M. J. Wingfield, K. A. Seifert & J. F. Webber), pp. 161–172. The American Phytopathological Society Press: St. Paul, Minnesota.

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: 11–136.

Hutchison LJ, Reid J. 1988a. Taxonomy of some wood-staining fungi from New Zealand 1. *Ophiostomataceae*. New Zealand Journal of Botany 26: 63–81.

Hutchison LJ, Reid J. 1988b. Taxonomy of some wood-staining fungi from New Zealand 2. Pyrenomycetes, Coelomycetes and Hyphomycetes. New Zealand Journal of Botany 26: 83–98.

Jacobs K, Kirisits T. 2003. *Ophiostoma kryptum* sp. nov. from *Larix decidua* and *Picea abies* in Europe, similar to *O. minus*. Mycological Research 107: 1231–1242.

Jacobs K, Wingfield MJ. 2001. *Leptographium* species: tree pathogens, insect associates, and agents of blue-stain. Academic Press, New York. 205 pp.

Jacobs K, Wingfield MJ, Wingfield BD. 2001. Phylogenetic relationships in *Leptographium* based on morphological and molecular characters. Canadian Journal of Botany 79: 719–732.

Kim J-J. 2001. Biological discoloration of radiata pine and its prevention. Ph.D. dissertation. Korea University, Seoul, Korea.

Kim J-J, Kim G-H. 2000. Mold and sapstain fungi associated with radiata pine logs imported from New Zealand. The International Research Group on Wood Preservation, Document No. IRG/WP 00-10346. Stockholm, Sweden.

Kim J-J, Kim SH, Lee S, Breuil C. 2003. Distinguishing *Ophiostoma ips* and *Ophiostoma montium*, two bark beetle-associated sapstain fungi. FEMS Microbiology Letters 222: 187–192.

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

Lee S, Kim J-J, Fung S, Breuil C. 2003. A PCR-RFLP marker distinguishing *Ophiostoma clavigerum* from morphologically similar *Leptographium* species associated with bark beetles. Canadian Journal of Botany 81: 1104–1112.

Lim YW, Alamouti SM, Kim J-J, Lee S, Breuil C. 2004. Multigene phylogenies of *Ophiostoma clavigerum* and closely related species from bark beetle-attacked *Pinus* in North America. FEMS Microbiology Letters 237: 89-96.

Price T. 1997. Elimination of sapstain in radiata pine logs exported from New Zealand. *In* Biology and prevention of sapstain, pp. 53-55. The Forest Products Society Press. Madison, Wisconsin.

Schroeder S, Kim SH, Chueng 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.

Spatafora JW, Blackwell M. 1994. Cladistic analysis of partial ssrDNA sequences among unitunicate perithecial Ascomycetes and its implications on the evolution of centrum development. *In* Ascomycete Systemtics – Problems and Persepectives in the Nineties (ed. D.L. Hawksworth), pp. 233–241. Plenum, New York.

Swofford DL. 2001. PAUP: *Phylogenetic analysis using parsimony. Version 4.0b10*. Sinauer Associates, Inc.: Sunderland, Mass.

Taylor JW, Jacobson DJ, Kroken S, Kasua T, Geiser DM, Hibbett DS, Fisher MC. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology 31: 21–32.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24: 4876–4882.

Upadhyay HP. 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis*. The University of Georgia Press, Athens, Georgia. 176 pp.

Vilgalys R, Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology 172: 4238–4246.

White TJ, Bruns TD, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* PCR protocols: a guide to methods and applications (eds. M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White), pp. 315-322. Academic Press, San Diego, California.

Wingfield MJ. 1993. *Leptographium* species as anamorphs of *Ophiostoma*: Progress in establishing acceptable generic and species concepts. *In Ceratocystis* and *Ophiostoma* taxonomy, ecology, and pathogenicity (eds. M. J. Wingfield, K. A. Seifert & J. F. Webber), pp. 43–52. The American Phytopathological Society Press: St. Paul, Minnesota.

Wingfield MJ, Capretti P, MacKenzie M. 1988. *Leptographium* spp. as root pathogen of conifers. An international perspective. *In Leptographium* root diseases on conifers (eds. T. C. Harrington & F. W. Cobb, Jr.), pp. 113–128. The American Phytopathological Society. St Paul, Minnesota.

Wingfield MJ, Gibbs JN. 1991. *Leptographium* and *Graphium* species associated with pine-infesting bark beetles in England. Mycological Research 95: 1257-1260.

Wingfield MJ, Seifert KA, Webber JF. 1993. *Ceratocystis* and *Ophiostoma* taxonomy, ecology, and pathogenicity. The American Phytopathological Society Press: St. Paul, Minnesota.

Zhou XD, de Beer, ZW, Harrington TC, McNew D, Kirisits T, Wingfield MJ. 2004. Epitypification of *Ophiostoma galeiformis* and phylogeny of species in the *O. galeiformis* complex. Mycologia 96: 1329-1338.