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Leptographium bistatum sp. nov., a new species with a *Sporothrix* synanamorph from *Pinus radiata* in Korea

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Radiata pine (*Pinus radiata*) lumber from Australia, Chile, and New Zealand is imported into Korea where it represents one of the most important sources of timber. Blue stain of this timber is a serious problem for which an integrated control strategy is being developed. One of the fungi that has been isolated from stained radiata pine sapwood in Korea is a *Leptographium* sp. that has a distinct *Sporothrix* synanamorph. The aim of this study was to classify this fungus. Morphological comparisons showed that this fungus is distinct from all other species of *Leptographium* and especially *L. elegans* and *L. francke-grosmanniae* that also have *Sporothrix* synanamorphs. Comparisons of sequence data for the ITS2 and part of the 28S rDNA genes as well as the β-tubulin gene also confirmed that this fungus represents an undescribed taxon, described here as *Leptographium bistatum* sp. nov.

INTRODUCTION

Species of *Leptographium* are common anamorphs of *Ophiostoma* and typically they have darkly coloured mononematous conidiophores terminating in several series of branches. The conidia are hyaline and form slimy masses at the apices of the conidiophore heads. These fungi are well known as associates of bark beetles (*Coleoptera: Scolytidae*) that infest mainly coniferous trees. Many species are weak pathogens and cause sap stain in dead trees and lumber. A small number of *Leptographium* spp. such as *Leptographium wageneri* are primary pathogens that are able to kill trees independently (Harrington & Cobb 1983, Wingfield, Capretti & Mackenzie 1988).

Radiata pine (*Pinus radiata*) is imported into Korea from Australia, Chile, and New Zealand and is the most important lumber source in Korea, accounting for about 60 % of the nation's lumber (Kim *et al*. 2002). Green *P. radiata* sapwood is highly susceptible to colonization by sapstain fungi, particularly in warm weather. As part of an integrated strategy for the control of wood discoloration, blue stain fungi occurring on *P. radiata* have been surveyed (Kim & Kim 2000, Kim *et al*. 2002). These surveys led to the discovery of an unidentified *Leptographium* species with a distinct *Sporothrix* synanamorph.

Sporothrix synanamorphs are common in *Pesotum* anamorphs of *Ophiostoma* (Upadhyay 1981, Seifert & Okada 1993). *Leptographium* spp. are essentially mononematous analogues of *Pesotum* and thus, the absence of *Sporothrix* synanamorphs in *Leptographium* has been considered unusual (Mouton, Wingfield & van Wyk 1992). *Leptographium* anamorphs of *Ophiostoma* species with *Sporothrix* synanamorphs have been reported only in *L. elegans* (Wingfield, Crous & Tzean 1994) and *O. francke-grosmanniae* (Davidson 1971, Mouton *et al*. 1992).

The aim of this study was to identify the *Leptographium* sp. with a distinct *Sporothrix* anamorph, associated with blue stain of *P. radiata* timber, imported into Korea. This was based on morphological features as well as on comparisons of DNA sequence data for the purported new species and other species considered to be closely related to it.

MATERIALS AND METHODS

Morphological and cultural studies

During the summer of 2001, five *Pinus radiata* logs imported from New Zealand to Korea were randomly

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selected at a local sawmill in Incheon, Korea. The logs were then cut and 50 freshly sawn sapwood boards, free of visible fungal colonization were stored in a mill yard warehouse to prevent direct exposure to sunlight. After one month, small pieces of blue stained sapwood were removed and placed on 2% DMEA (20 g Difco malt extract, 15 g Difco agar, and 1000 ml distilled water). Petri dishes were incubated at room temperature. Pure cultures for each fungal isolate used in this study were obtained using single spore isolation (Uzunovic *et al.* 2000).

The *Leptographium* sp. considered in this study had a distinct *Sporothrix* synanamorph. For this reason, it was specifically compared with the two other species of *Leptographium* known to have *Sporothrix* states. These were *L. elegans* (CMW 2245) and *O. franckegrosmanniae* (CMW 445). The fungus was also compared with *L. abieticolens* (CMW 2865) because both species had high similarity of the internal transcribed spacer 2 (ITS2) region and partial large subunit (LSU) rDNA sequences. All fungal cultures used in this study are maintained in the Korea University culture collection (KUC) and in the culture collection of the Department of Wood Science, University of British Columbia (Canada).

Cultures were grown and observed on 2% DMEA. Growth rates of cultures were determined at temperatures ranging between 5 and 35 °C, at five degree intervals. Agar disks (5 mm in diameter) taken from the edge of a freshly grown colony were placed at the centres of Petri dishes containing 2% DMEA medium, with three replicate plates for each test temperature. The colony diameters on each of the three replicate plates were measured along two perpendicular lines 3, 5, and 7 d after inoculation. Growth rates were calculated in mm per day. The tolerance of all the fungal isolates to cycloheximide was assessed by measuring growth at 25 ° on 2% DMEA amended with 0.05, 0.1, and 0.5% of cycloheximide.

Morphological features were observed on fungal structures produced on 2% DMEA and on sterile lodgepole pine (*Pinus contorta*) sapwood wafers. For light microscopy, fungal structures were mounted in water and observed using a Zeiss Axioplan light microscope. For scanning electron microscopy (SEM), small wood blocks ($10 \times 2 \times 7$ mm) were fixed using the method described by Lee *et al.* (2003). After fixation, samples were dried with a Balzers CPD 020 critical point drier. They were coated with gold palladium using a Nanotech Semprep II sputter coater and examined using a Hitachi S4700 scanning electron microscope.

PCR amplification of rDNA

For genomic DNA preparations, fungal isolates were inoculated on 2% OMEA (33 g Oxoid malt extract agar, 10 g Oxoid technical agar, and 1000 ml distilled water) media overlaid with sterile cellophane sheets (Bio-Rad) and grown for 7 d at room temperature. DNA extraction was carried out using the method described by Kim, Uzunovic & Breuil (1999).

The ITS2 region and partial LSU rDNA were amplified using the primers ITS3 and LR3 (Vilgalys & Hester 1990). The β-tubulin gene was amplified using the primer BT2E (5′-GTTCAYCTCCAGACCGCY-CAGTG-3′) and BT12 (Kim *et al.* 2003). PCR amplification was carried out with a total volume of 25 μl in 0.6 ml reaction tubes and a Touchdown Thermocycler (Hybaid). The reaction mix contained 1× reaction buffer (10 mM Tris-Cl [pH 8.0], 1.5 mM $MgCl_2$, 50 mM KCl), 80 μM of each deoxynucleotide, 20 pmol of each primer, 0.5U Taq DNA polymerase (Rose Scientific, Edmonton) and 100 ng genomic DNA. The standard reaction conditions were as follows: initial denaturation at 94 ° for 4 min, 30 cycles of denaturation at 94 ° for 50 s, primer annealing at 55 ° for 50 s and DNA elongation at 72 ° for 50 s, and a final cycle of DNA elongation at 72 ° for 10 min. PCR products were visualized by electrophoresis on a 1.4% agarose gel containing ethidium bromide.

DNA sequencing and phylogenetic analysis

The PCR products were gel-purified using a Qiaquick Gel Extraction Kit (Qiagen, Mississauga). Gel purified PCR products were sequenced using the primer ITS3 and LR3 (Vilgalys & Hester 1990, White *et al.* 1990). Sequencing was performed on an ABI 3700 automated sequencer (Perkin-Elmer, Foster City, CA) at the DNA Synthesis and Sequencing Facility, Macrogen (Seoul, Korea).

Sequences for other taxa used in comparisons were obtained from GenBank. The rDNA sequences of 28 taxa and the β-tubulin gene sequences of 14 taxa of ophiostomatoid fungi were aligned using CLUSTAL W, version 1.8 (Thompson, Higgins & Gibson 1994). Manual adjustment of the alignments was done in the PHYDIT program version 3.2 (http://plasza.snu.ac.kr/~jchun/phydit/). Ambiguous regions of rDNA and intron regions of the β-tubulin gene in the alignments were excluded from analyses. All parsimony analyses used the heuristic search option with simple addition sequences with MULPARS and TBR branch swapping. Gaps were treated as missing data. Branch stability was assessed by 1000 replicate parsimony bootstrap replications implemented with PAUP*4.0b10 (Swofford 2001).

TAXONOMY

The *Leptographium* isolated from *Pinus radiata* lumber in Korea and investigated in this study differs morphologically from all described species in this genus. The most obvious distinguishing characteristic of this fungus is the presence of a *Sporothrix* state that is considerably better developed than that in the two other *Leptographium* species, *L. elegans* and

the anamorph of *O. francke-grossmaniae*, which have *Sporothrix* synanamorphs. We, therefore, consider that this fungus represents a new taxon and provide the following description for it.

Leptographium bistatum J.-J. Kim & G.-H. Kim, **sp. nov.** (Figs 1–13)

Etym.: 'bistatum', referring to the two distinct anamorph states.

Coloniae creverunt optime ad 25 ° 3.4 mm die in 2% DMEA. Nullum incrementum videtur infra 10 °C Vel supra 35 °. In MEA cum 'cycloheximide', coloniae creverunt 3.4, 2.6 et 2.2 mm die in 0.05, 0.1 et 0.5%.

Coloniae in MEA effusae, extendentes, albae deinde olivaceofuscescentes. Hyphae in substrato submersae, mycelium aerium abundans, laete olivaceum, leve, 2–4 μm diametro. Conidiophora solitaria vel ad septeni aggregata, mononematosa, macronematosa cum rhizoideis. Stipites erecti, brunnei, simplices, 3–5-septati, 200–927 μm longi, basi 4.9–12.3 μm lati. Apparatus conidiogenus 42–69 μm longus, massa conidiali exclusa; ramis primariis usque ad tribus medio-brunneis, centralibus leviter maioribus quam aliis, 8–18 × 2.5–3 μm, ramis secondariis pallide brunneis, 5–8 ×2.5–3 μm, ramis tertiariis hyalinis, 2.5–5 × 2–2.5 μm. Cellulae conidiogenae discretae, apicem versus angustatae, 9.8–24.5 μm. Evolutio conidii per aedificationem parietis supplementariae ontogenia holoblastica et proliferatione percurrenti cum secessione retardata, ut falso videtur per proliferationem sympodialem. Conidia hyalina, oblonga vel ovoidea, apicibus rotundatis, basibus truncatis, medio distincte curvata, 3.5–6 × 1–2 μm; in massis hyalinis mucosis in apicibus conidiophorarum cumulantia. Synanamorphum *Sporothrix* cum hyphis hyalinis 1.5–2.5 μm latis formatum. Cellulae conidiogenae in mycelio dispersae, terminales vel in ramulis brevibus lateralibus, latitudine aequali vel in apice fertili tumido latissimae, 24–158 μm longae, 1–1.5 μm latae infra apicem tumidum protrusionis cylindricae. Conidia in denticulis sympodialiter portata, 3–4 μm longa, 1–1.5 μm lata. Conidia tumida denticulos distinctos gignentes cum conidiis secondariis in culturis vulgata.

Typus: **Korea**: *Incheon*: Sawmill, Jungsung Co., *Pinus radiata* lumber, 31 July 2001, *J.-J. Kim* (PREM 57692 – holotypus; KUC 2799, CMW 3802 – isotypi).

Growth. The optimal growth temperature for *Leptographium bistatum* was 25 ° with a growth rate of 3.4 mm d^{-1} on 2% DMEA. No growth was observed either below 10 ° or above 35 °. On DMEA amended with cycloheximide the mean growth rate of two isolates of *L. bistatum* was 3.4, 2.6, and 2.2 mm d^{-1} at concentrations of 0.05, 0.1 and 0.5%, respectively. On DMEA the colonies are effuse, spreading, white and then becoming greenish black (30F8; Kornerup & Wanscher 1961). *Hyphae* submerged in the medium and aerial mycelium abundant, greenish gray (27D2, Kornerup & Wanscher 1961), smooth, and 2–4 μm diam. *Conidiophores* single or in groups of up to seven, mononematous, macronematous with rhizoids at their bases. Stipes erect, brown, simple, 3–5 septate, 200–927 (mean = 607 ± 212) μm long, and 4.9–12.3 (mean = 8.1 ± 2.4) μm wide at the base. *Conidiogenous apparatus* 42–69 (mean = 55 ± 7.6) μm long excluding conidial mass; one to three medium brown primary branches, central branches slightly larger than the others, 8–18 (mean = 14.8 ± 2.4) × 2.5–3 μm; secondary branches pale brown, 5–8 (mean = 6.4 ± 1.5) × 2.5–3 μm; tertiary branches hyaline, 2.5–5 (mean = 3.8 ± 0.9) × 2–2.5 μm. *Conidiogenous cells* discrete, tapering distally, 9.8–24.5 (mean = 16.1 ± 3.8) μm long. *Conidium development* replacement wall building with holoblastic ontogeny and percurrent proliferation with delayed secession giving a false appearance of sympodial proliferation (Wingfield 1993). *Conidia* hyaline, oblong to ovoid, with rounded apices, truncate bases, and distinctly curved in the middle, 3.5–6 (mean = 4.4 ± 0.7) × 1–2 (mean = 1.5 ± 0.3) μm; accumulating in hyaline mucilaginous masses at the apices of the conidiophores.

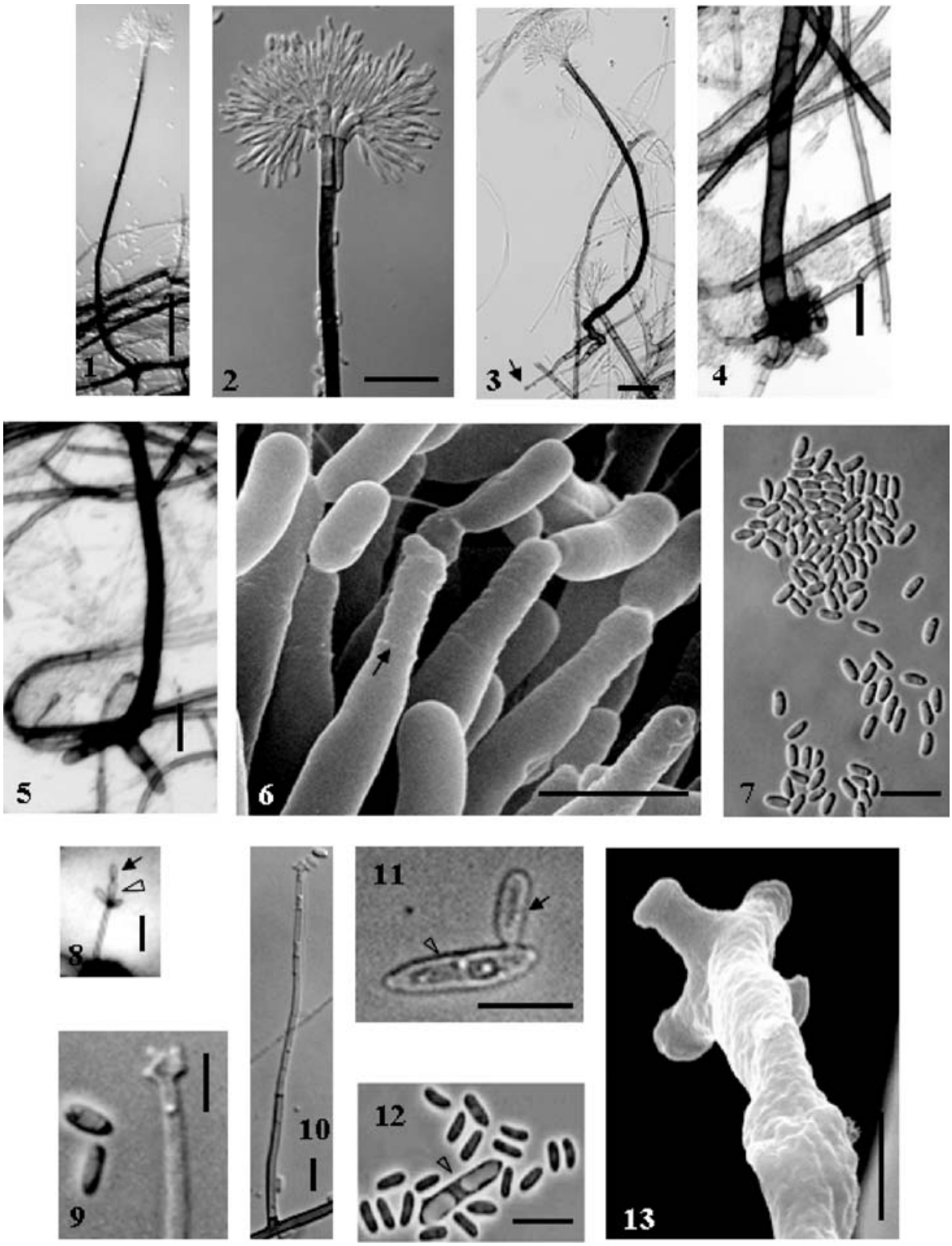
Synanamorph Sporothrix with hyaline hyphae, 1.5–2.5 μm wide. *Conidiogenous cells* scattered on mycelium, terminal or on short side branches, uniform width or widest at swollen fertile apex, 24–158 (mean = 63.6 ± 36.9) μm long, 1–1.5 (mean = 1.3 ± 0.1) μm wide below swollen apex of cylindrical protruding denticles. Conidia borne sympodially on denticles, 3–4 (mean = 3.4 ± 0.5) μm long and 1–1.5 (mean = 1.3 ± 0.2) μm wide. Swollen conidia, 6.5–13.5 (mean = 9.5 ± 2.0) × 2–3 (mean = 2.5 ± 0.4) μm long, giving rise to distinct denticles with secondary conidia common in cultures.

Additional specimens examined: **Korea**: *Incheon*: Sawmill, Jungsung Co., *Pinus radiata* lumber, 31 July 2001, *J.-J. Kim* (KUC 2804, CMW 3826, PREM 57693; *loc. cit*. (KUC 2798, 2800–2803).

RESULTS

The nucleotide sequences determined in this study have been deposited in GenBank DNA as follows: accession nos for *Leptographium bistatum* are AY348305 (KUC 2799)–AY348304 (KUC 2804) for the rDNA, and AY348306 (KUC 2799)–AY348307 (KUC 2804) for the β-tubulin gene. Other accession numbers of the β-tubulin gene used are AY348308 (*L. elegans* CMW 2245), AY348309 (*Ophiostoma francke-grosmanniae* CMW 445), and AY348310 (*L. abieticolens* CMW 2865).

After the introduction of gaps, the alignment of rDNA sequences included 642 nucleotide positions. Of these, 358 characters were constant, 124 were parsimony-uninformative, and 160 were parsimony-informative. Maximum parsimony analyses resulted in three equally parsimonious trees with a tree length 662; CI 0.6843; RI = 0.7515; RC 0.5142. In these trees, *L. bistatum* grouped with *L. abieticolens* and *O. francke-grosmanniae* (Fig. 14). The rDNA sequences of *L. bistatum* showed high similarity with the sequences of *L. abieticolens* (99.8%) and *O. francke-grosmanniae* (99.7%), but low similarity with *L. elegans* (84.4%). For the exons of the partial β-tubulin gene, the sequence similarity ranged from 89.8 to 91.6% within the ingroup taxa.



Figs 1–13. *Leptographium bistatum* (KUC2799). **Figs 1–7.** *Leptographium* synanamorph. **Fig. 1.** Light micrograph of a conidiophore. **Fig. 2.** Light micrograph of conidiogenous apparatus. **Fig. 3.** Light micrograph of conidiophore showing relatively short, septate stipe and with *Sporothrix* synanamorph with denticulate conidiogenous cells (arrow) at its base. **Figs 4–5.** Light micrograph of a mononematous conidiophore with rhizoids at base cell. **Fig. 6.** Scanning electron micrograph of conidiogenous cells showing annellations (arrow) and curved conidia. **Fig. 7.** Light micrographs of conidia. **Figs 8–13.** *Sporothrix* synanamorph. **Fig. 8.** Stereomicrograph of *Sporothrix* synanamorph with swollen conidia (ramoconidia-like) (open arrow) and secondary conidia (arrow). **Figs 9–10.** Light micrograph of *Sporothrix* synanamorph with denticulate conidiogenous cells. **Figs 11–12.** Light micrographs of swollen conidia (open arrows) and secondary conidia (arrow). **Fig. 13.** Scanning electron micrograph showing denticulate conidiogenous cell of *Sporothrix* synanamorph. Bars: Fig. 1 = 50 μm; Fig. 2 = 20 μm; Figs 3–5 = 10 μm; Fig. 6 = 2 μm; Fig. 7, 10–12 = 10 μm; Fig. 8 = 25 μm; Fig. 9 = 5 μm; Fig. 13 = 1 μm.

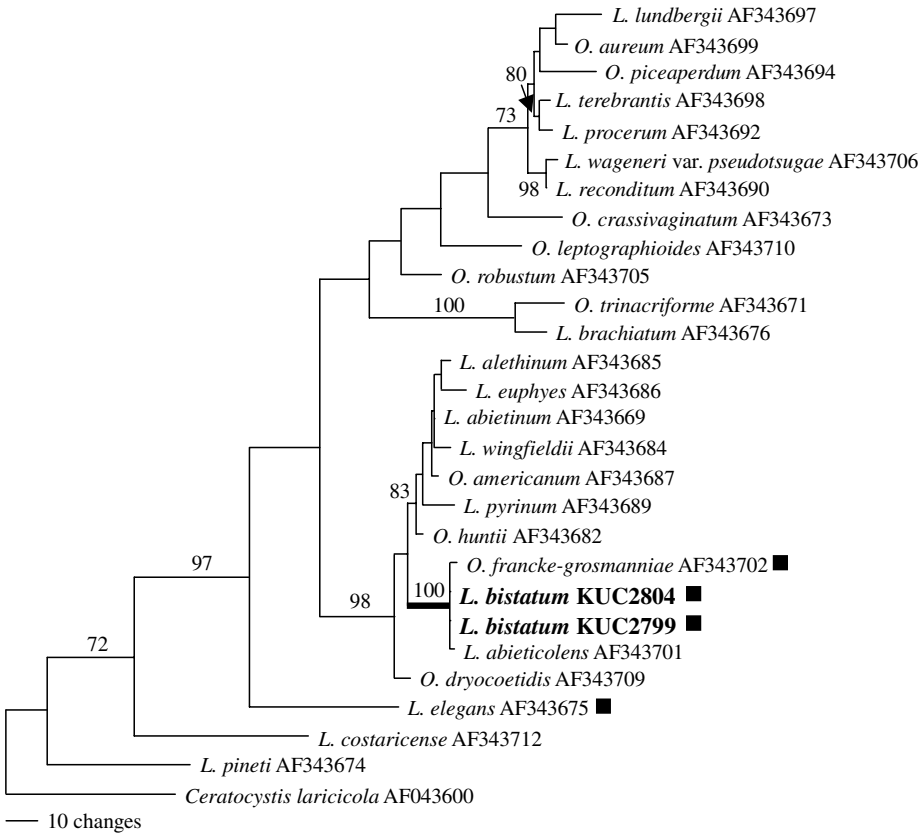


Fig. 14. One of three equally parsimonious trees obtained from analysis of ITS 2 region and partial large subunit region. Percentages from 1000 bootstrap replication are given above the branches (more than 70%) at the base of the corresponding clade. The tree was rooted with *Ceratocystis laricicola* as the outgroup. Fungal species having a *Sporothrix* synanamorph are indicated by black squares.

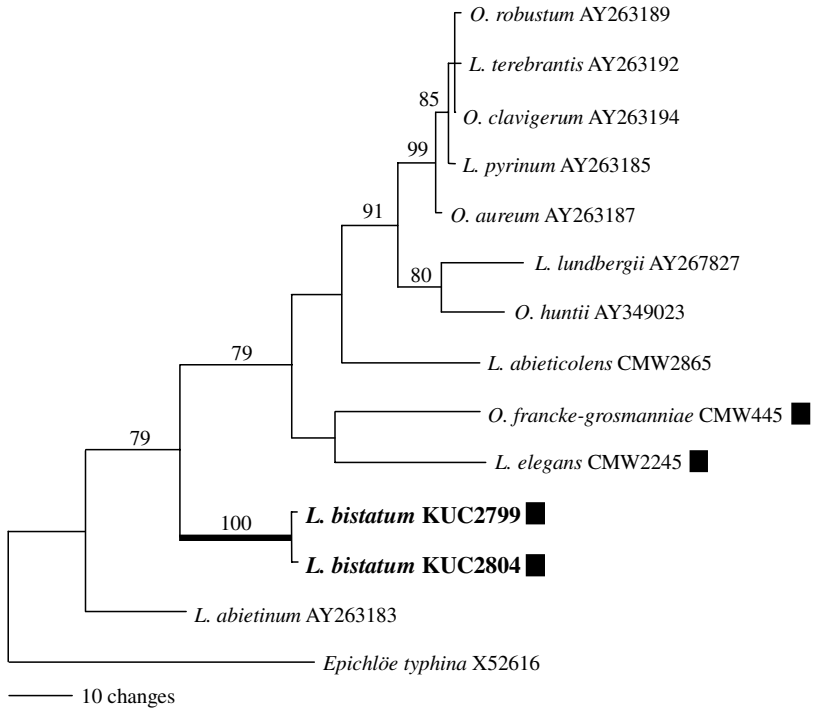


Fig. 15. The most parsimonious phylogenetic tree generated from the β-tubulin gene sequence. The tree was rooted with the sequence of *Epichlöe typhina* as the outgroup. The numbers above internodes indicate the bootstrap percentage from 1000 bootstrap replicates (for values greater than 70%). The black squares represent ophiostomioid fungi having a *Sporothrix* synanamorph.

The partial β-tubulin genes of *O. francke-grosmanniae* and *L. elegans* were shorter than in other species because their third introns were missing. When both exons and introns were included, the sequence similarity of the new species was 85.1% with *O. francke-grosmanniae*, 78.7% with *L. elegans* and 73.9% with *L. abieticolens*. When introns were excluded from the partial β-tubulin gene sequence for *L. bistatum*, 550 characters remained. The alignment resulted in 417 constant characters, of which 46 were parsimony-uninformative and 87 were parsimony-informative. Maximum parsimony analyses of this gene resulted in three equally parsimonious trees (tree length 266; CI 0.6805; RI = 0.6545; RC 0.4453) with essentially the same topology. In these trees, the two isolates representing *L. bistatum* did not constitute a monophyletic group with *O. francke-grosmanniae* and *L. elegans* (Fig. 15). However, the taxa consistently appeared in basal positions.

DISCUSSION

Results of this study have shown that the *Leptographium* isolated from blue-stained *Pinus radiata* lumber in Korea represents a distinct species. This fungus, named as *L. bistatum* here, has a well developed *Sporothrix* synanamorph, which represents its most obvious distinguishing characteristic. Two other *Leptographium* species, *L. elegans* and *L. francke-grosmanniae*, have *Sporothrix* synanamorphs (Wingfield *et al.* 1994, Jacobs & Wingfield 2001), but these are considerably less well developed than those of *L. bistatum*. Denticles on the *Sporothrix* conidiophores are distinctly cylindrical in side view and in *L. bistatum*, resemble those seen in the *Sporothrix* anamorphs of species such as *Ophiostoma piceae* and *Ophiostoma quercus* and thus unlike those of *L. elegans* and *O. francke-grosmanniae*.

Amongst the more important diagnositic characteristics of *Leptographium* spp. (Jacobs & Wingfield 2001), *L. bistatum* has very obvious curved conidia and well developed rhizoids at the bases of dark mononematous conidiophores. Two or more primary branches at the apices of these stipes represent Type B conidiophores (Jacobs & Wingfield 2001) without an obvious central branch. Peripherally, the conidia resemble those from species such as *L. abietinum* in which these spores are distinctly curved. The fungus, however, has a different ecology to *L. abietinum*. No described species of *Leptographium* has this combination of characteristics.

L. abieticolens, which is phylogenetically related to *L. bistatum*, originates from *Abies* species in eastern North America. This species is characterized by broadly ellipsoidal to obovoid conidia; has optimal growth on 2% MEA at 15 °; and hyphae are submerged or on top of agar with abundant aerial mycelium (Jacobs & Wingfield 2001). In contrast, *L. bistatum* has darker stipes and more complex conidiophores, an optimal growth temperature of 25 °, and the conidia have a very different shape to those of *L. abieticolens*.

Both *L. bistatum* and *O. francke-grosmanniae* have *Sporothrix* synanamorphs with denticulate conidiogenous cells. However, *O. francke-grosmanniae* produces perithecia on artificial media and wood (Davidson 1971), whereas *L. bistatum* appears to exist only in the anamorph form. The *Sporothrix* state in *O. francke-grosmanniae* is relatively rare and indistinct in cultures (Wingfield *et al.* 1994), while this state in *L. bistatum* is well developed and common. Furthermore, the conidiophores of *L. bistatum* are almost five times longer than those of *O. francke-grosmanniae* (Davidson 1971, Wingfield *et al.* 1994). The conidia of *L. bistatum* are also elongated and kidney shaped while those of *O. francke-grosmanniae* are oblong to obovoid.

L. elegans has a *Sporothrix* synanamorph and in this regard, it is also similar to *L. bistatum*. However, there are many morphological features that distinguish these two species. The conidiophores of *L. bistatum* are almost twice as long as those of *L. elegans* and *L. elegans* lacks rhizoids at the bases of its conidiophores (Wingfield *et al.* 1994), which are very distinct in *L. bistatum*. The oblong and oblong to obovoid conidia of *L. elegans* are also different to those of *L. bistatum*, which are distinctly curved. *L. elegans* was described from *Chamaecyparis formosensis*, a host very different to *P. radiata*. These fungi tend to be reasonably specific to groups of conifers (Jacobs & Wingfield 2001) and in terms of ecology they would not easily be confused.

Analysis of rDNA sequence data for *L. bistatum* showed that this fungus is distinct from *O. francke-grosmanniae* and *L. abieticolens*. Given their distinct morphological differences and differences in geographic and ecological origins, it was interesting that they had a relatively high level of rDNA sequence similarity. The β-tubulin gene phylogeny clearly reflected the relationships between *L. bistatum* and other species of *Leptographium*. *L. elegans* and *O. francke-grosmanniae* grouped together but this association had low bootstrap support and *L. bistatum* isolates formed a distinct clade with 100% bootstrap support. *L. abieticolens* grouped separately from those species with *Sporothrix* synanamorphs. Although the nuclear rDNA region containing ITS regions is useful for determining relationships between fungal genera and species (Bruns *et al.* 1992, O'Donnell 1992, LoBuglio, Pitt & Yaylor 1993), it does not permit distinguishing closely related taxa (Gams & Meyer 1998, Ospina-Giraldo *et al.* 1999, Hermosa *et al.* 2000, Harrington *et al.* 2001, Jacobs, Wingfield & Wingfield 2001). In this regard, sequences for the β-tubulin gene are apparently more useful. The phylogenetic analysis of the sequences for the β-tubulin gene not only clearly separated *L. bistatum* from *L. abieticolens* and *O. francke-grosmanniae*, but also separated these three species from other species lacking *Sporothrix* synanamorphs. The results of the

β-tubulin gene phylogeny, coupled with the morphological and cultural characteristics provide strong support for describing *L. bistatum* as a new species.

Leptographium spp. are well known associates of bark beetles (*Coloeoptera: Scolytidae*) that infest primarily conifers (Harrington 1988, Wingfield 1993). These insects transmit the fungi to timber and the fungi generally give rise to blue stain of the sapwood. It is most likely that *L. bistatum* has a bark beetle vector but the fact that it was isolated from blue stained wood precluded the identification of an associated insect. Two conifer infesting bark beetles, *Hylastes ater* and *Hylurgus ligniperda* are present in New Zealand, the origin of the *P. radiata* lumber considered in this study. These insects are native to Europe and various *Ophiostoma* and *Leptographium* spp. have been introduced into New Zealand with them (Harrington 1988, Wingfield *et al*. 1988, http://www.fs.fed.us/na/morgantown/fhp/palerts/red_haired_bark_beetle.pdf). These insects have been relatively well sampled in New Zealand (Bain 1977, Milligan 1978) and none of the *Leptographium* spp. associated with them resembles *L. bistatum*. We, therefore, believe that this fungus is of Korean origin and that it was transported to the *P. radiata* lumber after arrival in this country. Korea is importing logs from many tree species, including *P. radiata*, from Siberia, Southeast Asia and Oceania, and *L. bistatum* could have originated from these trees. Further studies are required to resolve this question.

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