Mycol. Res. **109** (3): 275–284 (March 2005). © The British Mycological Society
doi:10.1017/S0953756204002060 *Printed in the United Kingdom.*

A new *Leptographium* species associated with *Tomicus piniperda* infesting pine logs in Korea

Jae-Jin KIM[1], Young Woon LIM[1], Colette BREUIL[1]*, Michael J. WINGFIELD[2], Xu Dong ZHOU[2] and Gyu-Hyeok KIM[3]

[1] *Department of Wood Science, University of British Columbia, Vancouver, B C V6T 1Z4, Canada.*
[2] *Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0001, South Africa.*
[3] *Division of Environmental Science & Ecological Engineering, Korea University, Seoul 136-701, Korea.*
E-mail: breuil@interchg.ubc.ca

Received 24 August 2004; accepted 16 November 2004.

During the course of a survey of sapstaining fungi in Korea, a *Leptographium* was isolated from *Pinus densiflora* and *P. koraiensis* logs, infested with the bark beetle *Tomicus piniperda*. The fungus grew optimally at 25 °C on 2% malt extract agar and showed a high level of tolerance to cycloheximide. The *Leptographium* has unusually short conidiophores and is morphologically similar to *L. pini-densiflorae*, *L. lundbergii*, *L. yunnanense*, and the *Leptographium* anamorph of *Ophiostoma crassivaginatum*. Comparisons of DNA sequence data for three gene regions, as well as morphological characteristics, confirmed that this fungus represents an undescribed taxon. We consequently provide the name *Leptographium koreanum* sp. nov. for it here.

INTRODUCTION

Tomicus piniperda (*Coleoptera*: *Scolytidae*), a large pine shoot beetle, is an important forest pest that occurs naturally in Europe, North Africa, and Asia (including Siberia). In 1992, this insect was discovered in Ohio and later was reported in the northern and eastern USA well as in the southern part of Ontario and Québec (McCullough & Smitley 1995, Humphreys & Allen 1998, Masuya, Kaneko & Yamaoka 1998, http://creatures.ifas.ufl.edu/trees/beetles/Pine_shoot_beetle.htm). In Europe and North America, *T. piniperda* usually breeds in timber, recently killed trees, and weakened or stressed trees. However, the young adults feed on current or one-year-old lateral shoots, while the mature adults overwinter under the bark at the base of the same tree (Klepzig *et al.* 1991, Långström & Hellqvist 1991, 1993, Czokajlo *et al.* 1997). Despite the damage caused by *T. piniperda*, it is considered a secondary pest. However, in China native *T. piniperda* causes serious damage to *Pinus yunnanensis* and can kill healthy trees by mass attack (Ye 1991, Zhou *et al.* 2000, Lieutier, Ye & Yart 2003, Duan *et al.* 2004).

Like many other bark beetles, *T. piniperda* carries numerous species of *Ophiostoma* and their anamorphs, especially those in the genera *Leptographium*, *Pesotum* and *Sporothrix* (Solheim & Långström 1991, Wingfield & Gibbs 1991, Harrington 1988, Masuya *et al.* 1998). These fungi are best known as agents that stain the sapwood of conifers, and they are casually or specifically associated with insect vectors (Harrington 1988, Wingfield 1993). The ecological role of *Ophiostoma* spp. associated with conifer-infesting bark beetles is poorly understood and probably variable, depending on the insect concerned. Some species are highly pathogenic when inoculated into living trees and others cause a deep blue stain in the sapwood that reduces the value of the lumber (Harrington & Cobb 1983, Wingfield, Capretti & Mackenzie 1988, Solheim, Långström & Hellqvist 1993). For this reason, and as quarantine regulations seek to prevent the movement of *Ophiostoma* spp. between countries, it is important to identify them accurately.

Leptographium spp. have been relatively poorly collected, particularly in Asia. They are typified by dark erect conidiophores terminating in series of branches that give rise to conidiophores and conidia produced in slimy masses (Wingfield 1993, Jacobs & Wingfield 2001). They typically occur in the galleries of bark beetles where their spores easily attach to the bodies of pre-emergent insects. Due to their relatively simple morphology, and commonly overlapping features

* Corresponding author.

between species, they are well-known as being difficult to identify (Wingfield 1993, Jacobs, Wingfield & Wingfield 2001).

Several species of *Leptographium* have been isolated from *T. piniperda*. In Europe, these species are *L. guttulatum*, *L. lundbergii*, *L. procerum*, *L. wingfieldii*, *O. huntii* and *O. serpens* (Harrington 1988, Gibbs & Inman 1991, Solheim & Långström 1991, Wingfield & Gibbs 1991, Solheim *et al*. 1993, Jacobs & Wingfield 2001, Jacobs *et al*. 2001). In China, *L. yunnanense* is associated with *T. piniperda* that can infest healthy and non-stressed trees (Zhou *et al*. 2000) and *L. pinidensiflorae* (Masuya *et al*. 1998, 2000) has recently been associated with the insect in Japan. In Korea, *L. procerum* and an unidentified *Leptographium* sp. have been isolated from pitch pine (*Pinus rigida*) and Korean pine (*P. koraiensis*) logs (Oh 1999). The unidentified *Leptographium* sp. was subsequently collected during the course of a survey of sapstaining fungi on Korean pine and Japanese red pine (*P. densiflora*) logs, infested with *T. piniperda* (Kim, Kim & Ra 2002).

The unknown *Leptographium* sp. resembles *L. lundbergii* both morphologically and in terms of its habitat, and this name was tentatively used for it (Kim *et al*. 2001, 2002). However, preliminary comparisons of DNA sequence data have shown that the Korean fungus is different to other strains of *L. lundbergii*. The aim of this study was to re-consider the identity of the Korean *Leptographium* sp. associated with *T. piniperda*, using both morphological and physiological characteristics, as well as comparisons of DNA sequence data, including parts of the rDNA and protein coding genes. These studies also included comparisons with closely related *Leptographium* spp.

MATERIALS AND METHODS

Morphological and cultural studies

Korean and Japanese red pine trees were harvested and cut into logs during mid-January of 2000 and 2001. These logs were transported to three local sawmills where they were stacked in remote parts of the log yard. After approximately three months of storage, the logs were heavily infested with *Tomicus piniperda*.

For fungal isolations, small pieces of blue-stained sapwood were removed from the logs and placed on 2% MEA (20 g Difco malt extract, 15 g Difco agar, and 1000 ml distilled water). Petri dishes were incubated at room temperature, *ca* 20 °C. Pure cultures of each fungal isolate were obtained using a single conidial isolation technique (Uzunovic *et al*. 2000).

The *Leptographium* sp. was specifically compared with eight other species of *Leptographium* known to be associates of *T. piniperda* (Table 1). All fungal cultures used in this study are maintained in the Korea University Culture Collection (KUC) and in the Breuil culture collection (Department of Wood Science, University of British Columbia, Vancouver). Representative cultures of the *Leptographium* sp. from *T. piniperda* have also been deposited with the Centraalbureau voor Schimmelcultures (CBS, Utrecht).

Cultures were grown and observed on 2% MEA. Growth rates of cultures were determined at temperatures ranging from 5 and 35 °, at five degree intervals. Agar disks (5 mm diam), taken from the edge of a freshly grown colony, were placed at the centers of Petri dishes containing 2% MEA, with three replicate plates for each test temperature. The colony diameters on each of the three replicate plates were measured 3, 5, and 7 d after inoculation. Growth rates were calculated in $mm\ d^{-1}$. Cycloheximide tolerance of isolates was assessed by measuring growth at 25 ° on 2% MEA amended with 0.05, 0.1, and 0.5% of cycloheximide.

Morphological features were observed on fungal structures produced on 2% MEA and on sterile lodgepole pine (*Pinus contorta* var. *latifolia*) 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) bearing fungal structures 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.

DNA extraction, PCR amplification, sequencing and phylogenetic analysis

Isolates of various *Leptographium* spp. including the unknown species from Korea were used in DNA sequence comparisons (Table 1). DNA extraction was carried out using the method described by Kim, Uzunovic & Breuil (1999). The internal transcribed spacer (ITS) 2 and partial large subunit (LSU) regions of the ribosomal DNA operon were amplified using the primers ITS3 and LR3 (Vilgalys & Hester 1990, White *et al*. 1990). Portions of the actin and β-tubulin genes were amplified using the primers Lepact F /Lepact R (Lim *et al*. 2004) and T10 (O'Donnell & Cigelnik 1997)/ BT12 (Kim *et al*. 2003), respectively. 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, ON). Purified PCR products were sequenced using the same primer sets described above. Sequencing was performed on an ABI 3700 automated sequencer (Perkin-Elmer, Foster City, CA) at the DNA synthesis and Sequencing Facility, Macrogen (Seoul).

Sequences for the taxa other than those that were specifically derived in this study, were obtained from GenBank. The rDNA sequences of 29, the actin gene sequences of 16, and the β-tubulin gene sequences of 24

Table 1. *Leptographium* and *Ophiostoma* cultures used in this work and GenBank accession numbers for sequences.

Species	Isolate[a]	GenBank accession no.[b]			Host	Origin	Source[d]	Collector
		Actin	β-tubulin	ITS 2 and LSU				
L. koreanum	KUC 2078	AY707174	AY707183	AY707196	*Pinus densiflora*	Bongwha, Korea	Log infested with *TP*	*J.-J. Kim & G.-H. Kim*
	KUC 2102	AY707175	AY707184	AY707197	*P. koraiensis*	Yeoju, Korea	Log infested with *TP*	*J.-J. Kim & G.-H. Kim*
L. lundbergii	UAMH 9584	(AY544585)	(AY263184)	(AY544603)	*P. sylvestris*	Uppland, Sweden	Board	*A. Mathiesen-Käärik*
	CMW 217	AY707176	AY707185	AY707198	*P. sylvestris*	Sweden	Unknown	*T. Lagerberg/E. Melin*
L. pini-densiflorae	CMW 5157	–	AY707186	AY707199	*P. densiflora*	Japan	Tree infested with *TP*	*H. Masuya*
	CMW 5158	–	AY707187	AY707200	*P. densiflora*	Japan	Tree infested with *TP*	*H. Masuya*
L. procerum	CMW 12	–	–	(AF343692)	*P. strobus*	NY, USA	Unknown	*M. J. Wingfield*
	C 83[c]	–	–	AY707201	*Pseudotsuga menziesii*	ID, USA	Unknown	*T. C. Harrington*
L. serpens	CMW 60	–	AY707188	AY707202	*Pinus pinaster*	Tokai, South Africa	Unknown	*M. J. Wingfield*
	CMW 2844	–	AY707189	AY707203	*P. pinaster*	Tokai, South Africa	Unknown	*M. J. Wingfield*
L. wingfieldii	CMW 2095	AY707177	AY707190	AY707204	*P. strobus*	France	Tree infested with *TP*	*M. Morelet*
	CMW 2096	AY707178	AY707191	AY707205	*P. sylvestris*	France	Tree infested with *TP*	*M. Morelet*
L. yunnanense	CMW 5304	AY707179	AY707192	AY707206	*P. yunnanensis*	Vimen, China	*TP*	*Z. Xudong*
	CMW 5152	AY707180	AY707193	AY707207	*P. yunnanensis*	Vimen, China	*TP*	*Z. Xudong*
O. huntii	UAMH 4997	(AY544599)	(AY349023)	(AY544617)	*P. contorta*	Canada	Tree infested with *DP*	*R. C. R. Jeffrey*
	CBS 398.77	AY707181	AY707194	AY707208	*P. monticola*	Canada	Log	*R. W. Davidson*
O. piceaperdum	C 274[c]	AY707182	AY707195	AY707209	*Picea rubens*	USA	Unknown	*T. C. Harrington*

[a] CMW, Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria; KUC, Korean University Culture Collection, Korean University; UAMH, University of Alberta Microfungus Collection and Herbarium, Devonian Botanic Garden, Edmonton; C, Collection of T. C. Harrington, Iowa State University; CBS, Centraalbureau voor Schimmelcultures, Utrecht.

[b] Accession nos of sequences obtained from GenBank presented in parentheses.

[c] Provided by A. Uzunovic.

[d] *TP*, *Tomicus piniperda*; and *DP*, *Dendroctonus ponderosae*.

Ophiostoma spp. or their anamorphs were aligned using CLUSTAL X (Thompson *et al*. 1997). Manual adjustment of the alignments was done with the PHYDIT program version 3.2 (http://plasza.snu.ac.kr/~jchun/phydit/). Unalignable regions of rDNA were excluded and intron regions from the two protein coding genes were also excluded. Gaps were treated as missing data. Maximum parsimony trees (MPTs) were identified by heuristic searches using the tree bisection reconnection (TBR) branch-swapping algorithm. All characters were of equal weight and unordered. Concordance of three different data sets was evaluated using the partition homogeneity test implemented with PAUP*4.0b10, using 1000 replicates and the heuristic general search option (Huelsenbeck, Bull & Cunningham 1996). Phylogenetic analyses of the sequence data for individual loci and the three loci combined were performed with PAUP*4.0b10 (Swofford 2002). Branch stability was assessed by 1000 replicate parsimony bootstrap replications implemented with PAUP*4.0b10 (Swofford 2002).

RESULTS

DNA sequence data and phylogenetic analyses

The nucleotide sequence data, generated in this study have been deposited in GenBank DNA sequence database (Table 1). The alignment of rDNA sequences, after the introduction of gaps, generated 631 nucleotide positions; 17 ambiguous characters were excluded from the analyses. Only 65 characters were parsimony-informative, while 69 were parsimony-uninformative and 480 were constant. Maximum parsimony analyses resulted in 13 equally parsimonious trees (tree length of 194; CI 0.8505; RI 0.8497) because their sequences were very similar; one is shown in Fig. 7a. Topological differences among the 13 MPTs were due to branching order changes of a few taxa. In these trees, the *Leptographium* sp. associated with *Tomicus piniperda* in Korea was grouped with *L. yunnanense*, *L. lundbergii* and *Ophiostoma huntii*. The rDNA sequences of the unidentified *Leptographium* species showed high similarity to the sequences of *L. yunnanense* (99.5%) and *L. lundbergii* (99.5%). Although the branch in the tree separated the *Leptographium* sp. from other closely related species, it was not supported by bootstrap analysis (Fig. 7a).

Partial actin gene sequences for most of the taxa included in this study had two exons and one intron. The β-tubulin gene sequences possessed three exons and introns. When both exons and introns were included, the β-tubulin sequence similarity of the unidentified *Leptographium* sp. was 98.41–98.54% with *L. yunnanense*, 92.81 with *O. huntii* and 92.44 with *L. lundbergii*. When introns were excluded from partial actin and β-tubulin gene sequences, 667 and 550 characters remained, respectively. Most of the polymorphic positions were found in the synonymous third position. Maximum parsimony analyses of these genes resulted in 12 MPTs for the β-tubulin gene (tree length 248; CI 0.6613; RI 0.7717) and 2 MPTs for the actin gene (tree length 60; CI 0.8667; RI 0.9223) with essentially the same topology. One of the MPTs for each of the β-tubulin and actin gene datasets is shown in Fig. 7b and c, respectively. In these trees, the two isolates representing the unidentified *Leptographium* sp. from Korea constituted a distinct clade alongside *L. yunnanense*, *L. lundbergii* and *O. huntii*.

To resolve the clades with less ambiguity, a combined dataset comprised of rDNA and two protein coding genes was analyzed with *O. piceaperdum* as an outgroup. Results of the partition-homogeneity test ($P=0.718$) indicated that there was no significant conflict between the datasets and that three different gene trees reflected the same underlying phylogeny. Maximum parsimony analyses of the combined dataset generated one MPT (tree length 163; CI 0.8773; RI 0.9301). The phylogeny of the combined dataset was similar to the phylogenetic trees derived for the individual genes (Fig. 7d). Overall, there was increased bootstrap support when the combined dataset was used. The unidentified *Leptograhium* sp. associated with *T. piniperda* in Korea resided in a distinct group with strong basal support (97%), showing a sister relationship with *L. yunnanense*.

Morphology

The *Leptographium* sp. isolated from logs of Korean and Japanese red pine infested with *Tomicus piniperda* was typical of the genus, having well-developed erect conidiophores and masses of condia carried in moist drops at their apices. The fungus was able to tolerate high concentrations of cycloheximide, a typical feature of the *Ophiostoma* species and their anamorphs. The *Leptographium* sp. was most similar to *L. yunnanense* and the *Leptographium* anamorph of *O. crassivaginatum*. It was, however, morphologically distinct from these species (Table 2). Based on its phylogenetically distinct nature, and its distinct morphological characteristics, the *Leptographium* sp. from blue-stained *P. koraiensis* and *P. densiflora* infested with *T. piniperda* in Korea represents a new taxon, formally described here.

TAXONOMY

Leptographium koreanum J.-J. Kim & G.-H. Kim, **sp. nov.** (Figs 1–6)

Etym.: *koreanum*, referring to the fungus being first collected in Korea.

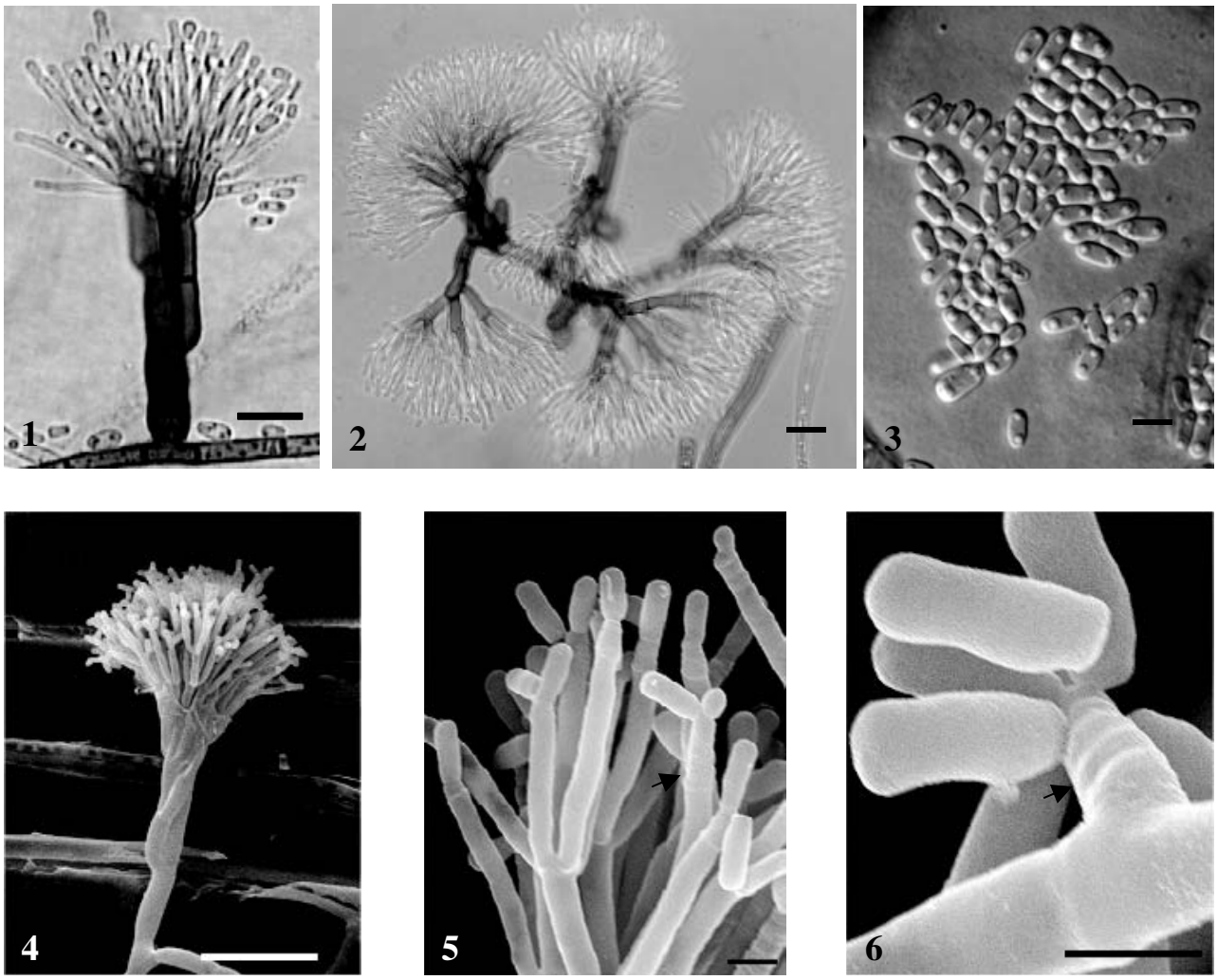
Crescit optime ad 25 °C, tum 17 mm per diem in 2% DMEA. Non crescit infra 5 ° vel supra 35 °. In MEA cum alio 0.05, 0.1 et 0.5 gl^{-1} 'cycloheximide', crescit ad 25 ° alium 16, 14 et 8 mm per diem.

Coloniae in MEA effusae, extendentes, albae olivaceo-brunnescentes. Hypharum parietes laeves, hyphae 2–4 μm

Table 2. Characteristics of *Leptographium koreanum* and morphologically similar species.

	L. koreanum	*L. lundbergii*[a]	*L. pini-densiflorae*[b]	*L. yunnanense*[c]	*O. crassivaginatum*[d]
Region	Korea	North America, New Zealand, Europe, South Africa, Japan,	Japan	China	Canada
Host	*Pinus densiflora*, *P. koraiensis*	*Pinus sylvestris*, *P. densiflora*, *P. taeda*, *P. radiata*, *P. ponderosa*, *P. strobus*, *Pinus* spp., *Picea abies*, *Picea* spp., *Larix leptolepis*	*Pinus densiflora*	*Pinus yunnanensis*, *P. gaoshanesis*, *P. shimaonensis*	*Picea mariana*, *Picea glauca*, *P. resinosa*, *P. strobus*, *P. sylvestris*, *Populus grandidentata*, *Populus tremuloides*, *Fraxinus nigra*
Insect association	*T. piniperda*	Many other bark beetles including *T. piniperda*	*T. piniperda*, *T. minor*, *Xyleborus validus*	*T. piniperda*	*Trypodendron retusus*
Stipe length	35–227 μm	(35–)214.5–306.5(–635) μm	32–190(–324) μm	11–66(–112) μm	8.0–60(–85) μm
Conidiogenous apparatus length	24–79 μm	(35–)42.5–85(–150) μm	22–80 μm	(40–)83–88(–127) μm	15.5–56.5(–62) μm
Conidium shape	Oblong to ovoid, Relatively straight	Broadly ellipsoid with Prominently truncate ends	Oblong to ellipsoid, Sometimes clavate	Oblong to obovoid	Oblong to obovoid Distinctly curved
Conidium length	3–10 μm	3–5 μm	2.6–8.3(–13) μm	(4–)7–8(–11) μm	(4–)4.5–5.5(–10) μm
Conidium width	1.5–3.5 μm	2–4 μm	0.9–3 μm	2–6 μm	1–2.5 μm
Teleomorph	Absent	Absent	Absent	Absent	*Ophiostoma*
Rhizoids	Absent	Absent	Present	Absent	Absent
Optimum temperature for growth	25 °	20 °	25 °	25 °	30 °
Growth rate (diam)[e]	17 mm day^{-1} at 25 °C 13 mm day^{-1} at 20 °C	5.6 mm day^{-1} at 25 ° 13 mm day^{-1} at 20 °	*ca* 12 mm day^{-1} at 25 ° 6.4 mm day^{-1} at 20 °	11 mm day^{-1} at 25 °	3.6 mm day^{-1} at 30 °
Granular hyphae	Absent	Absent	Absent	Present	Present

[a] Jacobs & Wingfield (2001) and Masuya *et al*. (2000).
[b] Jacobs & Wingfield (2001) and Masuya *et al*. (2000).
[c] Jacobs & Wingfield (2001) and Zhou *et al*. (2000).
[d] Jacobs & Wingfield (2001).
[e] Growth rate of *L. yunnanense* was re-examined in this study because it was not correctly reported in the original paper (Zhou, pers comm.).



Figs 1–6. *Leptographium koreanum* (KUC2078). **Fig. 1.** Light micrograph of a conidiophore. **Fig. 2.** Light micrograph of conidiophores grouped. **Fig. 3.** Light micrograph of conidia. **Fig. 4.** Scanning electron micrograph of a conidiophore. **Figs 5–6.** Scanning electron micrographs of conidiogenous cells showing annellations (arrows). Bars: Fig. 1 = 20 μm; Fig. 2 = 10 μm; Fig. 3 = 5 μm; Fig. 4 = 25 μm; Figs 5–6 = 2 μm.

diametro. Conidiophorae singulae vel ad quinae aggregatae, mononematae, macronematae, interdum in hyphis aeriis factae. Stipites erecti, brunnei, simplices, 3–11-septati, 35–227 (medius 89 ± 43) μm longi, basi 3–8 (medius 5 ± 1.5) μm lati. Apparatus conidiogenus 24–79 (medius = 50.7 ± 13) μm longus, massa conidiali exclusa, ramis primariis singulis vel ternis medio-brunneis, ramis centralibus aegre quam aliis maioribus, 8–16 (medius 13 ± 3) × 2.5–3 μm; ramis secondariis laete brunneis, 5–13 (9 ± 3) × 2.5–3 μm; ramis tertiariis hyalinis, 4–11 (8 ± 2) × 2–2.5 μm. Cellulae conidiogenae discretae, apicem versus angustatae, 6–11 (medius 10 ± 1.5) μm longae. 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 distinctis truncatis 3–10 (medius 5 ± 2) × 1.5–3.5 (medius 2 ± 0.5) μm; in massis hyalinis mucosis in apicibus conidiophorarum cumulantia.

Typus: **Korea**: *Yeoju*: Sawmill, Central Forest Products Processing & Marketing Center, *Pinus koraiensis* log, 10 Aug. 2000, *J.-J. Kim & G.-H. Kim* (PREM 58256-holotypus; KUC 2102, CMW 14199-isotypi).

Optimal growth temperature 25 °, with a growth rate of 17 mm day^{-1} on 2% DMEA. No growth was found below 5 ° or above 35 °. On MEA amended with 0.05, 0.1, and 0.5 gl^{-1} cycloheximide, the mean growth rates of three isolates at 25 ° were 16, 14, and 8 mm d^{-1}, respectively. *Colonies* effuse, spreading, white becoming olive-brown on MEA, (4F8; Kornerup & Wanscher 1961). *Hyphae* smooth-walled, 2–4 μm wide. *Conidiophores* single or in groups of up to five, mononematous, macronematous without rhizoids at their bases, sometimes produced on aerial hyphae. *Stipes* erect, brown, simple, 3–11 septate, 35–227 (mean = 89 ± 43) μm long, and 3–8 (mean = 5 ± 1.5) μm wide at the base. *Conidiogenous apparatus* 24–79 (mean = 50.7 ± 13) μm long excluding conidial mass; one to three medium brown primary branches, central branches slightly larger than the others, 8–16 (mean 13 ± 3) × 2.5–3 μm; secondary branches pale brown, 5–13 (9 ± 3) × 2.5–3 μm; tertiary branches hyaline, 4–11 (8 ± 2) × 2–2.5 μm. *Conidiogenous cells* discrete, tapering distally, 6–11 (mean = 10 ± 1.5) μm long. Conidium

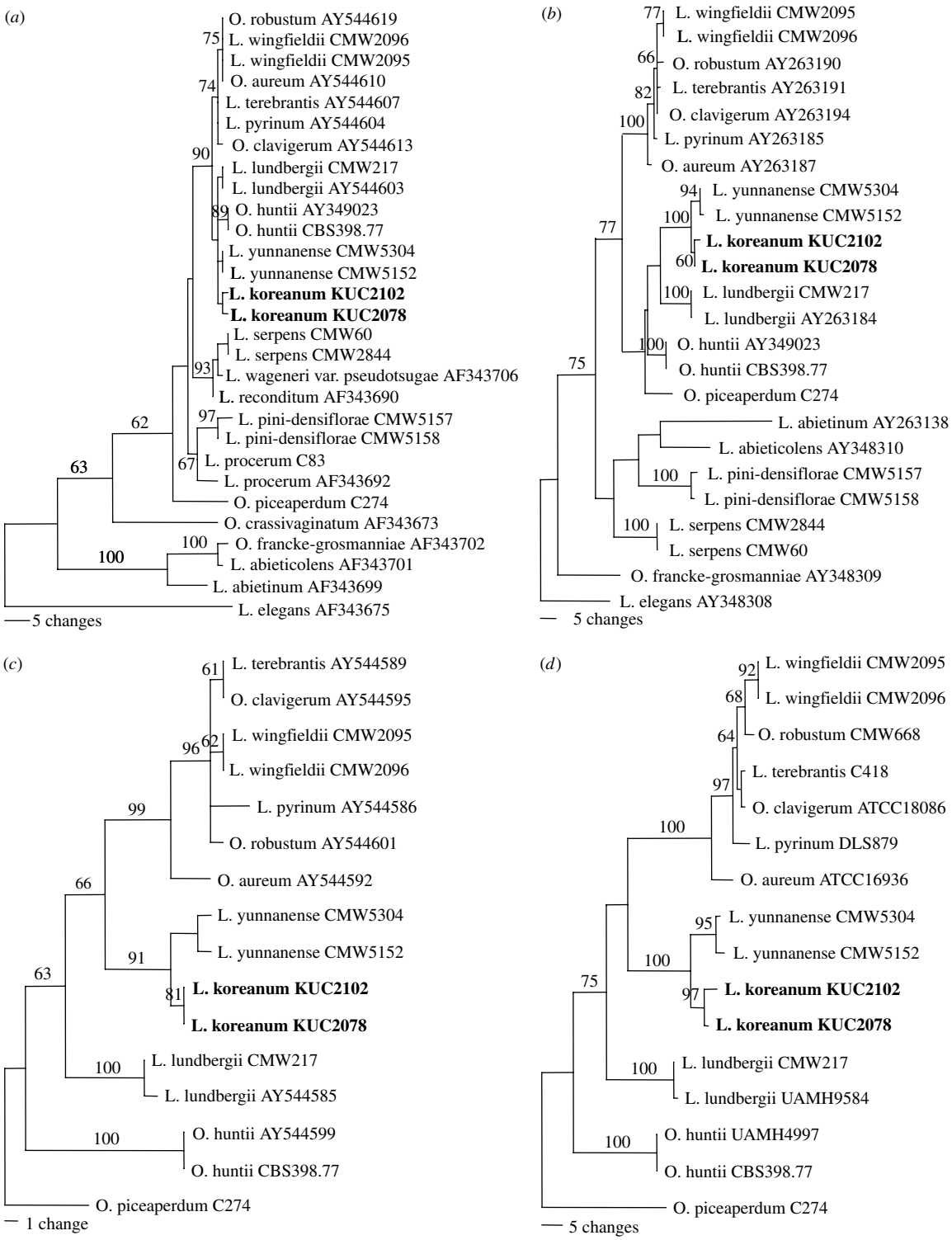


Fig. 7. Most parsimonious trees for each of the three nuclear gene datasets and the combined datasets: (*a*) rDNA, (*b*) β-tubulin, (*c*) actin, and (*d*) combined. Numbers above or below the branches are bootstrap values of MPT shown when greater than 60%.

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, distinct truncate bases, 3–10 $(\text{mean} = 5 \pm 2) \times 1.5$–$3.5$ $(\text{mean} = 2 \pm 0.5)$ µm; accumulating in hyaline mucilaginous masses at the apices of the conidiophores.

Additional specimens examined: **Korea**: *Bongwha*: Sawmill, National Forestry Cooperatives Federation, *Pinus densiflora* log, 9 Aug 2000, *J.-J. Kim & G.-H. Kim* (KUC 2078, CMW 14201, PREM 58261); *loc.cit.* (KUC 2072, CMW 14200,

PREM 58257); *loc.cit*. (KUC2071, KUC2073–2077); *loc.cit*., *P. densiflora* lumber, May 2001, *J.-J. Kim & G.-H. Kim* (KUC2031–2310); *loc.cit*., *P. densiflora* disc, July 2001, *J.-J. Kim & G.-H. Kim* (KUC2501–2510); *Yeoju*: Sawmill, Central Forest Products Processing & Marketing Center (CFPP&MC), *P. koraiensis* log, 10 Aug 2000, *J.-J. Kim & G.-H. Kim* (KUC 2101, KUC2103–2105); *Gapyeong*: Sawmill, CFPP & MC, *P. koraiensis* lumber, May 2001, *J.-J. Kim & G.-H. Kim* (KUC2401–2410); *Gapyeong*: Sawmill, CFPP & MC, *P. koraiensis* disc, July 2001, *J.-J. Kim & G.-H. Kim* (KUC2601–2610).

DISCUSSION

Results of this study have shown that the unknown *Leptographium* sp. associated with *Tomicus piniperda* infestation of Japanese red and Korean pine logs represents an undescribed taxon, named here as *L. koreanum*. This new species adds to a relatively large number of *Ophiostoma* and *Leptopgraphium* spp. that have been associated with *T. piniperda* in various parts of the world (Harrington 1988, Solheim & Långström 1991, Wingfield & Gibbs 1991, Masuya *et al*. 1998, Jacobs & Wingfield 2001).

L. koreanum is morphologically most similar to *L. pini-densiflorae* and *L. lundbergii*. The latter two species were also those critically compared by Masuya *et al*. (2000) in the course of describing a new *Leptographium* from *T. piniperda* in Japan. *L. koreanum* can easily be distinguished from *L. pini-densiflorae* based by the presence of rhizoid-like structures at the base of the conidiophores in the latter species (Masuya *et al*. 2000). It is likewise possible to distinguish between *L. lundbergii* and *L. koreanum* based on the following characteristics. *L. lundbergii* is characterized by its prominently truncate conidia, with sizes between $3–5 \times 2–4$ μm, and its conidiophores that are (90–) 246–409 (–685) μm long (Jacobs & Wingfield 2001). In contrast, *L. koreanum* has shorter conidiophores with conidia that are about twice the size of those in *L. lundbergii*. The very small conidiophores of *L. koreanum* represent an important morphological characteristic of this species and are produced abundantly in artificial cultures on a solid surface.

Other than morphological features, *L. koreanum* can be distinguished from *L. pini-densiflorae* and *L. lundbergii* based on their optimum temperatures for growth and their growth rates at 20 °. *Leptographium pini-densiflorae* and *L. lundbergii* grow best at 25 and 20 °, respectively (Masuya *et al*. 2000). Like *L. pini-densiflorae*, *L. koreanum* grows also best at 25 °. However, when grown at 20 °, *L. koreanum* and *L. lundbergii* have the same growth rate of about 13 mm d^{-1}, while *L. pini-densiflorae* isolates grow much more slowly at about 6.4 mm day^{-1} (Masuya *et al*. 2000). This is approximately half the growth rate of *L. koreanum*.

Short conidiophores similar to those of *L. koreanum* are characteristic of *L. yunnanense* and the *Leptographium* anamorph of *O. crassivaginatum*. Like *L. koreanum*, *L. yunnanense* has been isolated from pine trees infested with *T. piniperda*. In contrast to *L. koreanum*, the two other species have short robust conidiophores and a granular sheath material around their hyphae, as reported for *L. pyrinum* (Zhou *et al*. 2000, Jacobs & Wingfield 2001). In addition, the conidiophores of *L. koreanum* are more discrete with tightly formed apical branches, very unlike the loosely arranged branches that form the conidiogenous apparatus of *L. crassivaginatum* and *L. yunnanense* (Zhou *et al*. 2000, Jacobs & Wingfield 2001). The conidia of *L. yunnanense* are approximately the same length as those of *L. koreanum* but they are almost twice as wide in the former species. Although the conidia in *L. koreanum* are of a similar length to those of *L. crassivaginatum*, conidia in the latter species are distinctly curved, as opposed to the relatively straight conidia in the former species (Jacobs & Wingfield 2001). In addition to these differences, *L. koreanum* grows four times faster than the other two species on 2% MEA at 25 ° (Zhou *et al*. 2000, Jacobs & Wingfield 2001).

Although nuclear rDNA regions including ITS regions are useful for determining the relationships between fungal genera and species (Bruns *et al*. 1992, O'Donnell 1992, Jacobs *et al*. 2001), they often do not distinguish between closely related taxa (Gams & Meyer 1998, Ospina-Giraldo *et al*. 1999, Hermosa *et al*. 2000, Harrington *et al*. 2001, Jacobs *et al*. 2001). In this study, over 99% sequence similarities were shown between various *Leptographium* spp. including *L. lundbergii*, *L. koreanum*, *O. huntii*, *L. pyrinum*, *L. terebrantis*, *L. wingfieldii*, *O. aureum*, *O. clavigerum* and *O. robustum*. These results indicate that the rDNA region including the ITS2 region and partial LSU rDNA are inappropriate to differentiate *L. koreanum* from these closely related species. In contrast, we were able to use partial sequences of the actin and β-tubulin genes to distinguish between *Leptographium* spp. (Lee *et al*. 2003, Kim *et al*. 2004, Lim *et al*. 2004) including those considered in this study. Separate phylogenies for the partial actin and β-tubulin gene sequences as well as the combined phylogenies clearly reflected the relationships between the newly described *L. koreanum* and other species of *Leptographium*. Although *L. pini-densiflorae* and *O. crassivaginatum* are morphologically similar to *L. koreanum*, DNA sequence comparisons showed that these species are very distantly related. Our phylogenetic analyses clearly separated *L. koreanum* from its closest relative, *L. yunnanense*. The two species were further separated from related species including, *L. lundbergii*, *O. huntii*, and *L. pini-densiflorae*. These DNA sequence comparisons as well as distinct morphological characteristics provide robust support for describing *L. koreanum* as a new taxon.

Leptographium spp. are well known associates of bark beetles (*Coloeoptera*: *Scolytidae*) that, primarily, infest conifers (Harrington 1988, Wingfield 1993). These insects transmit the fungi to the sapwood, which

most of these fungi discolour. In our survey of sap-staining fungi, *L. koreanum* was frequently isolated from pine logs attacked by *T. piniperda*. Because *T. piniperda* has also been found to carry various other *Leptographium* spp., it was interesting that this was the only *Leptographium* sp. associated with it in our study.

Very little is known regarding the pathogenicity of *Leptographium* spp. associated with bark beetles. Only one species, *L. wageneri* is known to be a primary pathogen (Harrington & Cobb 1983, Harrington 1988) but various other species have been shown to display high levels of pathogenicity when inoculated into healthy trees. In this regard, one of the most pathogenic species of *Leptographium* is *L. wingfieldii* (Solheim *et al.* 1993, Solheim, Krokene & Långström 2001). Like *L. koreanum*, this fungus is carried by *T. piniperda* and it is believed to contribute to tree death in Europe (Solheim & Långström 1991, Solheim *et al.* 1993, 2001). *L. wingfieldii* has recently been recognized as one of the fungi that had been introduced into North America with *T. piniperda* (Jacobs *et al.* 2004). It would be equally important to assess the relative pathogenicity of *L. koreanum* and inoculations on healthy trees are planned to achieve this goal.

It is particularly interesting that *T. piniperda* carries distinctly different *Leptographium* spp. in different countries. One possibility is that the insects in geographically disparate countries represent distinct species. In this regard, *T. piniperda* from the Yunnan Province of China consistently carries *L. yunnanense*, which, for example, is unlike *T. piniperda* in Europe that is closely associated with *L. wingfieldii* (Wingfield & Gibbs 1991, Zhou *et al.* 2000). Recent studies based on DNA sequence comparisons have shown that *T. piniperda* in the Yunnan Province of China represents a species very similar to but distinct from *T. piniperda* in Europe (Lieutier *et al.* 2003, Duan *et al.* 2004). Similar findings have been reported for other bark beetles. Thus, it is possible that the Korean *T. piniperda* differs from those species occurring elsewhere in the world, and that the differences observed in their fungal associates might reflect small differences in the insects.

ACKNOWLEDGEMENTS

This work was supported by the Agricultural R&D Promotion Center of Korea and by the Natural Sciences and Engineering Research Council of Canada (C. B.). We also thank Hugh Glen (National Botanic Gardens, Durban, South Africa) for assistance in preparing the Latin diagnosis.

REFERENCES

Bruns, T. D., Vilgalys, R., Barns, S. M., Gonzles, D., Hibbett, D. S., Lane, D. J., Simon, L., Stickel, S., Szaro, T. M., Weisberg, W. G. & Sogin, M. L. (1992) Evolutionary relationships within the fungi: analyses of nuclear small subunit rRNA sequences. *Molecular Phylogenetics and Evolution* **1**: 231–241.

Czokajlo, D., Wink, R. A., Warren, J. C. & Peale, S. A. (1997) Growth reduction of Scots pine, *Pinus sylvestris*, caused by the larger pine shoot beetle *Tomicus piniperda* (Coleoptera: Scolytidae), in the New York state. *Canadian Journal of Forest Research* **27**: 1394–1397.

Duan, Y., Kerdelhue, C., Ye, H. & Lieutier, F. (2004) Genetic study of the forest pest *Tomicus piniperda* (*Col., Scolytinae*) in Yunnan province (China) compared to Europe: new insights for the systematics and evolution of the genous *Tomicus*. *Heredity* **93**: 416–422.

Gams, W. & Meyer, W. (1998) What exactly is *Trichoderma harzianum*? *Mycologia* **90**: 904–915.

Gibbs, J. N. & Inman, A. (1991) The pine shoot beetle *Tomicus piniperda* as a vector of blue-stain fungi to windblown pine. *Forestry* **64**: 239–249.

Harrington, T. C. (1988) *Leptographium* species, their distributions, hosts and insect vectors. In *Leptographium Root Diseases on Conifers* (T. C. Harrington & F. W. Cobb jr., eds): 1–39. American Phytopathological Society Press, St Paul, MN.

Harrington, T. C. & Cobb, F. W. jr. (1983) Pathogenicity of *Leptographium* and *Verticicladiella* species isolated from roots of western North American conifers. *Phytopathology* **73**: 596–599.

Harrington, T. C., 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.

Hermosa, M. R., Grondona, I., Monte, E., Iturriaga, E. A., Diaz-Minguez, J. M., Castro, C., Monte, E. & Garcia-Acha, J. M. (2000) Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. *Applied and Environmental Microbiology* **66**: 1890–1898.

Huelsenbeck, J. P., Bull, J. J. & Cunningham, C. W. (1996) Combining data in phylogenetic analysis. *TREE* **11**: 152–158.

Humphreys, N. & Allen, E. (1998). *The Pine Shoot Beetle*. [Exotic Forest Pest Advisory No 2.] Natural Resources Canada, Pacific Forestry Centre, Victoria, BC.

Jacobs, K., Bergdahl, D. R., Wingfield, M. J., Halik, S., Seifert, K. A., Bright, D. E. & Wingfield, B. D. (2004) *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* **108**: 411–418.

Jacobs, K. & Wingfield, M. J. (2001) *Leptographium Species: Tree Pathogens, Insect Associates and Agents of Blue-Stain*. Academic Press, New York.

Jacobs, K., Wingfield, M. J. & Wingfield, B. D. (2001) Phylogenetic relationships in *Leptographium* based on morphological and molecular characters. *Canadian Journal of Botany* **79**: 719–732.

Jacobs, K., Wingfield, M. J., Coetsee, C., Kirisits, T. & Wingfield, B. D. (2001) *Leptographium guttulatum* sp. nov., a new species from spruce and pine in Europe. *Mycologia* **93**: 380–388.

Kim, G.-H., Kim, J.-J. & Ra, J.-B. (2002) Development of fungal sapstain in logs of Japanese red pine and Korean pine. *Journal of the Korean Wood Science and Technology* **30**: 128–133.

Kim, J.-J., Lim, Y. W., Wingfield, M. J., Breuil, C. & Kim, G.-H. (2004) *Leptographium bistatum* sp. nov., a new species with a *Sporothrix* synanamorph from *Pinus radiata* in Korea. *Mycological Research* **108**: 699–706.

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

Kim, J.-J., Ra, J.-B., Son, D.-S. & Kim, G.-H. (2001) Fungi colonizing sapwood of Japanese red pine logs in storage. *Microbiology* **29**: 205–209.

Kim, S. H., 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.

Klepzig, K. D., Raffa, K. F. & Smalley, E. B. (1991) Association of an insect-fungal complex with red pine decline in Wisconsin. *Forest Science* **37**: 111–1139.

Kornerup, A. & Wanscher, J. H. (1961) *Methuen Handbook of Colour*. Methuen, London.

Långström, B. & Hellqvist, C. (1993) Induced and spontaneous attacks by pine shoot beetles on young Scots pine trees: tree mortality and beetle performance. *Journal of Applied Entomology* **115**: 25–36.

Långström, B. & Hellqvist, C. (1991) Shoot damage and growth losses following three years of *Tomicus* attacks in Scots pine stands close to timber storage site. *Silva Fennica* **25**: 133–45.

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.

Lieutier, F., Ye, H. & Yart, A. (2003) Shoot damage by *Tomicus* sp. (*Coleoptera*: *Scolytidae*) and effect on *Pinus yunnanensis* resistance to subsequent reproductive attacks in the stem. *Agricultural and Forest Entomology* **5**: 227–233.

Lim, Y. W., Alamouti, S. M., 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.

Masuya, H., Kaneko, S. & Yamaoka, Y. (1998) Blue stain fungi associated with *Tomicus piniperda* (*Coleoptera*: *Scolytidae*) on Japanese red pine. *Journal of Forest Research* **3**: 213–219.

Masuya, H., Wingfield, M. J., Kaneko, S. & Yamaoka, Y. (2000) *Leptographium pini- densiflorae* sp. nov. from Japanese red pine. *Mycoscience* **41**: 425–430.

McCullough, D. C. & Smitley, D. R. (1995) Evaluation of insecticides to reduce maturation feeding by *Tomicus piniperda* (*Coleoptera*: *Scolytidae*) in scotch pine. *Journal of Economic Entomology* **88**: 693–699.

O'Donnell, K. (1992) Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). *Current Genetics* **22**: 213–220.

O'Donnell, K. & Cigelnik, E. (1997) Two divergent intragenomic rDNA ITS 2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* **7**: 103–116.

Oh, E. S. (1999) *Studies on the cultural characteristics of blue stain fungi isolated in Korea and their biological control*. MSc thesis, Kangwon National University, Korea.

Ospina-Giraldo, M. D., Royse, D. M., Chen, X. & Romanie, C. P. (1999) Molecular phylogenetic analyses of biological control strains of *Trichoderma harzianum* and other biotypes of *Trichoderma* spp. associated with mushroom green mold. *Phytopathology* **89**: 308–313.

Solheim, H., Krokene, P. & Långström, B. (2001) Effects of growth and virulence of associated blue-stain fungi on host colonization behaviour of the pine shoot beetles *Tomicus minor* and *T. piniperda*. *Plant Pathology* **50**: 111–116.

Solheim, H. & Långström, B. (1991) Blue-stain fungi associated with *Tomicus piniperda* in Sweden and preliminary observations on their pathogenicity. *Annales des Science Forestieries* **48**: 149–156.

Solheim, H., Långström, B. & Hellqvist, C. (1993) Pathogenicity of the blue-stain fungi *Leptographium wingfieldii* and *Ophiostoma minus* to scots pine: effect of tree pruning and inoculum density. *Canadian Journal of Forest Research* **23**: 1438–1443.

Swofford, D. L. (2002) *PAUP: phylogenetic analysis using parsimony*. Version 4.0b10. Sinauer Associates, Sunderland, MA.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**: 4876–4882.

Uzunovic, A., Seifert, K. A., Kim, S. H. & Breuil, C. (2000) *Ophiostoma setosum*, a common sapwood staining fungus from western North America, a new species of the *Ophiostoma piceae* complex. *Mycological Research* **104**: 486–494.

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, T. J., Bruns, T. D., Lee, S. & Taylor, J. W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 315–322. Academic Press, San Diego.

Wingfield, M. J. (1993) *Leptographium* species as anamorphs of *Ophiostoma*: Progress in establishing acceptable generic and species concepts. In *Ceratocystis and Ophiostoma Taxonomy, Ecology, and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 43–52. American Phytopathological Society Press, St Paul, MN.

Wingfield, M. J., Capretti, P. & Mackenzie, M. (1988) *Leptographium* spp. as root pathogens of conifers. An international perspective. In: *Leptographium Root Diseases on Conifers* (T. C. Harrington & F. W. Cobb jr., eds): 113–128. American Phytopathological Society Press, St Paul, MN.

Wingfield, M. J. & Gibbs, J. N. (1991) *Leptographium* and *Graphium* species associated with pine-infesting bark beetles in England. *Mycological Research* **95**: 1257–1260.

Ye, H. (1991) On the bionomy of *Tomicus piniperda* (L.) (*Col. Scolytidae*) in the Kunming region of China. *Journal of Applied Entomology* **112**: 366–369.

Zhou, X. D., Jacobs, K., Morelet, M., Ye, H., Lieutier, F. & Wingfield, M. J. (2000) A new *Leptographium* species associated with *Tomicus piniperda* in southwestern China. *Mycoscience* **41**: 573–578.

Corresponding Editor: D. L. Hawksworth