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Leptographium wingfieldii introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles

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Leptographium wingfieldii is a well-known fungal associate of the pine shoot beetle, *Tomicus piniperda*, in Europe. This fungus is pathogenic to pines and is an important cause of blue-stain in the sapwood of infested trees. *Tomicus piniperda* was first found in a Christmas tree plantation in Ohio, USA, 1992, but isolation of the fungi associated with these intercepted insects was not attempted. Fungal strains resembling *L. wingfieldii* were recently isolated from pines attacked by *T. piniperda*, *Dendroctonus valens* and *Ips pini* in the northeastern United States. These strains were morphologically similar to the ex-type and other reference strains of *L. wingfieldii*. Strains were also compared based on sequences of the partial ITS ribosomal DNA operon, β-tubulin and elongation factor 1-alpha (EF-1α) genes. Based on these DNA sequence comparisons, reference strains of European *L. wingfieldii* were conspecific with North American strains from pines attacked by *T. piniperda*, *D. valens* and *I. pini*. A single strain from Canada, collected in 1993 near the Ontario border with the USA, shortly after the discovery of *T. piniperda* in that area and tentatively identified as *L. wingfieldii*, was also included in this study. Its identification was confirmed, suggesting that *L. wingfieldii* has been present in this region and probably over the whole range of the insect's distribution for at least a decade. This represents the first record of *L. wingfieldii* associated with the introduced and damaging pine shoot beetle *T. piniperda* in North America. It shows that the fungus is well established and can become associated with other native bark beetles that attack stressed and/or dying trees. The occurrence and spread of this highly pathogenic fungus associated with North American bark beetles should be monitored.

INTRODUCTION

The bark beetle *Tomicus piniperda* (*Coleoptera*: *Scolytidae*) is native to Europe and Asia where it infests the stems of stressed pine trees (Speight 1980). This insect is normally considered a secondary colonizer of the stems of trees, but is a primary pest on the terminal shoots where it undergoes maturation, especially in the growing tips. These shoots become hollow, die and can be recognized by the brown foliage and resin holes near the shoot bases (Speight 1980).

Tomicus piniperda was recently introduced into North America where it was first collected in 1992, predominantly on exotic Scots pine (*Pinus sylvestris*) near Cleveland, Ohio (Haack & Kucera 1993, Haack, Lawrence & Heaton 1993). The insect was subsequently found on native jack (*P. banksiana*), red (*P. resinosa*) and white (*P. strobus*) pine as well as on the exotic Austrian pine (*P. nigra*) (Haack *et al*. 1993). *Tomicus piniperda* spread rapidly in the USA and was soon found in a number of states in the eastern USA and southern Canada. It was discovered in Ontario in 1993, and has subsequently spread throughout southern Ontario and parts of Quebec (Bright 1996, Canadian Food Inspection Agency 2001, Morgan, de Groot & Smith 2002). The insect was first collected from insect traps in Vermont in 1999 (data not shown).

In Europe, *T. piniperda* is associated with several blue stain fungi (Morelet 1988, Gibbs & Inman 1991, Solheim & Långström 1991, Wingfield & Gibbs 1991). The most common of these are *Ophiostoma minus* and *Leptographium wingfieldii*. Both these fungi are pathogenic and are thought to contribute to rapid tree death (Lieutier *et al*. 1989, Solheim & Långström 1991, Solheim, Långström & Hellqvist 1993, Solheim, Krokene & Långström 2001). However, *L. wingfieldii*

Table 1. Strains of *Leptographium wingfieldii* used in the study.

Strain	Isolate number	GenBank accession no.	Date of isolation	Origin	Host	Associated insect	Collector
L. wingfieldii	CMW 2095	AY553400[a], AY534948[b], AY536194[c]	1987	Europe	*Pinus strobus*	*Tomicus piniperda*	*M. Morelet*
	CMW 2096	AY553398[a], AY534946[b], AY536192[c]	1987	Europe	*P. sylvestris*	*T. piniperda*	*M. Morelet*
North American strains	CMW 2019	AY553399[a], AY534947[b], AY536193[c]	1993	Ontario, Canada	*P. sylvestris*	*T. piniperda*	*Don Bright*
	CMW 10221	AY553404[a], AY534952[b], AY536198[c]	Oct. 2000	Vermont, USA	*P. strobus*	*Dendroctonus valens*	*D. R. Bergdahl*
	CMW 10223	AY553412[a], AY534960[b], AY536206[c]	Sept. 2000	Vermont, USA	*P. resinosa*	*D. valens*	*D. R. Bergdahl*
	CMW 10224	AY553401[a], AY534949[b], AY536195[c]	May 2001	Michigan, USA	*P. sylvestris*	*Tomicus piniperda*	*D. R. Bergdahl*
	CMW 10225	AY553405[a], AY534953[b], AY536199[c]	May 2001	Michigan, USA	*P. sylvestris*	*T. piniperda*	*D. R. Bergdahl*
	CMW 10226	AY553406[a], AY534954[b], AY536200[c]	June 2001	Vermont, USA	*P. sylvestris*	*Ips pini*	*D. R. Bergdahl*
	CMW 10227	AY553407[a], AY534955[b], AY536201[c]	June 2001	Vermont, USA	*P. sylvestris*	*I. pini*	*D. R. Bergdahl*
	CMW 10228	AY553408[a], AY534956[b], AY536202[c]	June 2001	Vermont, USA	*P. sylvestris*	*I. pini*	*D. R. Bergdahl*
	CMW 10229	AY553409[a], AY534957[b], AY536203[c]	June 2001	Vermont, USA	*P. sylvestris*	*I. pini*	*D. R. Bergdahl*
	CMW 10230	AY553410[a], AY534958[b], AY536204[c]	June 2001	Vermont, USA	*P. sylvestris*	*I. pini*	*D. R. Bergdahl*
	CMW 10232	AY553403[a], AY534951[b], AY536197[c]	May 2001	Michigan, USA	*P. sylvestris*	*Tomicus piniperda*	*D. R. Bergdahl*
	CMW 10237	AY553411[a], AY534959[b], AY536205[c]	Jan. 2002	Michigan, USA	*P. sylvestris*	*T. piniperda*	*D. R. Bergdahl*
	CMW 10238	AY553402[a], AY534950[b], AY536196[c]	Jan. 2002	Michigan, USA	*P. sylvestris*	*T. piniperda*	*D. R. Bergdahl*
L. terebrantis	CMW 9	AY553384[a], AY534932[b], AY536178[c]	1980	Minnesota, USA	*P. sylvestris*	*Hylobius pales*	*M. J. Wingfield*
	CMW 10216	AY553385[a], AY534933[b], AY536179[c]	Sept. 2000	Vermont, USA	*P. strobus*	*Dendroctonus valens*	*D. R. Bergdahl*
	CMW 10217	AY553386[a], AY534934[b], AY536180[c]	Sept. 2000	Vermont, USA	*P. strobus*	*D. valens*	*D. R. Bergdahl*

[a] ITS2 and partial 28S gene; [b] Partial β-tubulin gene; [c] Partial Elongation 1-α gene.

is more pathogenic and elicits long lesions and blue-stained sapwood in inoculation experiments on *P. sylvestris* (Solheim *et al*. 1993, 2001).

L. wingfieldii is a typical *Leptographium* species, with penicillately branched conidiophores and conidia that accumulate in slimy masses at the apices of conidiophores (Jacobs & Wingfield 2001). *L. wingfieldii* can be recognized by its elongated conidiophores, which are slightly yellow compared to the olivaceous conidiophores of most other *Leptographium* species. Also, conidia of *L. wingfieldii* can be distinguished by the wide variation in its conidial sizes and shapes, from smaller oblong conidia to much larger often clavate conidia (Jacobs & Wingfield 2001).

Although *T. piniperda* is well recognized as a serious and potentially damaging exotic invader species in North America, very little is known of the fungi it may have introduced into this new region. To date, only native species of stain fungi have been isolated from *T. piniperda* (Haack & Lawrence 1997). *Ophiostoma minus* already occurs both in Europe and North America (Münch 1907, Nelson 1934, Bramble & Holst 1940, Solheim & Långström 1991, Uzunovic *et al*. 1999, Gorton & Webber 2000), and a new introduction of *O. minus* into North America would not easily be detected. However, *L. wingfieldii* was previously unknown in North America and its introduction could have important pathological and ecological consequences.

Recently, strains of *Leptographium* spp. were collected from *P. sylvestris* attacked by *T. piniperda* in Michigan and from *P. strobus* and *P. sylvestris* infested with *Dendroctonus valens* and *Ips pini* beetles in Michigan and Vermont. The objective of this study was to identify these fungi to determine whether those associated with *T. piniperda* might be *L. wingfieldii*. We also investigated a single strain tentatively identified as *L. wingfieldii* collected in Niagara Falls, Ontario, Canada in 1993, shortly after *T. piniperda* was first found in southern Ontario (Bright 1996).

MATERIALS AND METHODS

Strains used in the study

Fungal strains examined in this study were isolated from mature bark beetles at sites in Burlington and Derby in Vermont and from *Tomicus piniperda* collected in the Crow and Snowcap areas in Michigan in 2001–02 (Table 1). Also, strains were isolated from the lower stems of *Pinus strobus* and *P. resinosa* trees attacked by *Dendroctonus valens*, and *P. sylvestris* attacked by *T. piniperda* (Michigan) and *I. pini* (Derby, Vermont) between Sept. 2000 and Jan. 2002. These strains were compared with the ex-holotype strain of *L. wingfieldii* (CMW 2096) and an additional reference strain of *L. wingfieldii* (CMW 2095) from Europe. The Canadian specimen was collected on *P. sylvestris* bolts in the Niagara Parks Commission area, Niagara Falls,

Ontario near the Ontario-New York State border in May 1993 (Bright 1996). The original specimen is deposited as DAOM 232221 (=CMW 2019). All strains are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria (Table 1), and representative isolates have also been deposited with the Centraalbureau voor Schimmelcultures (CBS, Utrecht).

Morphological studies

All measurements and microscopic observations were made from fungal structures grown on 2% Malt Extract agar (MEA) (20 g Biolab Malt extract, 20 g Agar, 1000 ml distilled water) and Oatmeal agar (OA) (Gams, Hoekstra & Aproot 1998) and incubated in incident light at 25 °C. Fungal structures were mounted on slides in 85% lactic acid and examined using phase or differential interference contrast microscopy.

Phylogenetic analyses

Strains (Table 1) were grown on commercial Potato Dextrose agar (PDA) for 10 d at 25 °. DNA extractions from pure cultures were prepared using the method of Möller *et al.* (1992). Mycelium was ground with TES extraction buffer (100 mM Tris, 10 mM EDTA, 2% SDS) and 100 µg proteinase K in 2 ml Eppendorf tubes and submerged in liquid nitrogen. The mixture was immediately incubated at 60 ° for 60 min. After incubation, 140 µl NaCl (5 M) and 65 µl CTAB was added and the mixture was incubated for 10 min at 65 °. SEVAG (24:1 chloroform:isoamylalcohol) was added in a 1:1 ratio and the mixture was incubated for a further 30 min at 0 °. Cell debris was removed from the mixture by centrifugation at maximum speed for 10 min. The supernatant was transferred to clean 1.5 ml Eppendorf tubes and 0.55 V cold isopropanol was added followed by centrifugation at maximum speed for 10 min. The supernatant was discarded and the pellet washed twice with 70% ethanol. The pellet was then dissolved in 100 µl sterile water. Successful isolation of DNA was confirmed on 1% agarose gels stained with ethidium bromide.

Amplification of the ITS2 and part of the large subunit (28S) of the rDNA operon, as well as partial β-tubulin and elongation factor 1-α (EF 1-α) genes was achieved using standard protocols for PCR reactions. PCR reactions were performed in 25 µl volumes containing 2.5 mM $MgCl_2$, 1 × PCR buffer, 0.2 mM dNTP, 0.2 mM of each primer and 2.5 U Taq-polymerase enzyme. Primers used in the amplification reactions and for cycle sequencing were ITS3 and LR3 (White *et al.* 1990) for the ITS2 and 28S region and Bt2a and Bt2b (Glass & Donaldson 1995) for the β-tubulin gene. Fungal specific primers were designed for the EF 1-α gene. In order to design the primers, degenerate primers, designed by Rehner (2001) based on the EF 1-α gene of *Puccinia graminis*, were used to amplify a part of the EF 1-α gene. These primers were EF1-526F [(VNVVVIGHVD) 5′-GTCGTYGTYATYGGHCAYGT-3′] and EF1-1567R [(rev/comp of KIGGIGTV): 5′-ACHGTRCCRATACCACCRATCTT-3′] (http://www.nacse.org/~faaberg/aftol/EF1primer.pdf). From these sequences, specific primers were designed that amplify and sequence the last part of the second exon to the first part of the last exon and include 2 variable introns [EF1F (5′-TGCGGTGGTATCGACAAGCGT-3′) and EF2R (5′-AGCATGTTGTCGCCGTTGAAG-3′)]. PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden) and sequenced using the Big Dye terminator cycle sequencing premix kit (Applied Biosystems, Foster city, CA) on an ABI PRISM 310 automatic sequencer (Perkin Elmer Applied Biosystems, CA). Sequence contigs were assembled using Sequence Navigator (Applied Biosystems), alligned in ClustalX (Thompson *et al.* 1997) and manually adjusted in PAUP* v.4.0b10 (Swofford 2001). Bases from the 3′ end of the 5.8S region and the 5′ end of the large ribosomal subunit were excluded from the analysis of the ITS data in order to align the data set.

For all data sets, phylogenetic relationships were inferred using heuristic searches in PAUP* v.4.0b10 (Swofford 2001). Characters were treated as unweighted in the analysis and gaps were treated as a fifth base. Heuristic searches were performed with tree-bisection-reconnection (TBR) branch swapping. Starting trees were obtained through stepwise addition. *Cryphonectria cubensis* was used as outgroup (Fig. 1). The resulting trees were used to obtain a consensus tree. Confidence levels were estimated using a Bootstrap analysis (1000 replicates) with the 'fast'-stepwise addition option. For the β-tubulin and EF 1-α genes, ambiguously aligned regions were coded and step matrices for assigning weight to the codes were computed using INAASE 2.3b (Lutzoni *et al.* 2000). The weighted codes were included in the analysis, replacing the ambiguous aligned regions. To determine whether the three data sets could be combined, a Templeton Nonparametric Wilcoxon Signed Ranked Test was performed (Kellogg *et al.* 1996).

A Bayesian Markov Monte Carlo analysis (Larget & Simon 1999) was performed to test the confidence for the tree nodes in the parsimony analysis. The analysis was performed using MrBayes 2.01 (Heulsenbeck & Ronquist 2001). The algorithm ran for 500 000 generations and every tenth tree was sampled. Four cold chains were run simultaneously and the first 3500 trees were discarded as the burnin period, to avoid using trees generated before convergence of the Markov chains.

RESULTS

Morphology and ecology

The strains from *Pinus sylvestris* attacked by *Tomicus piniperda* and *Ips pini*, and *P. resinosa* and *P. strobus*

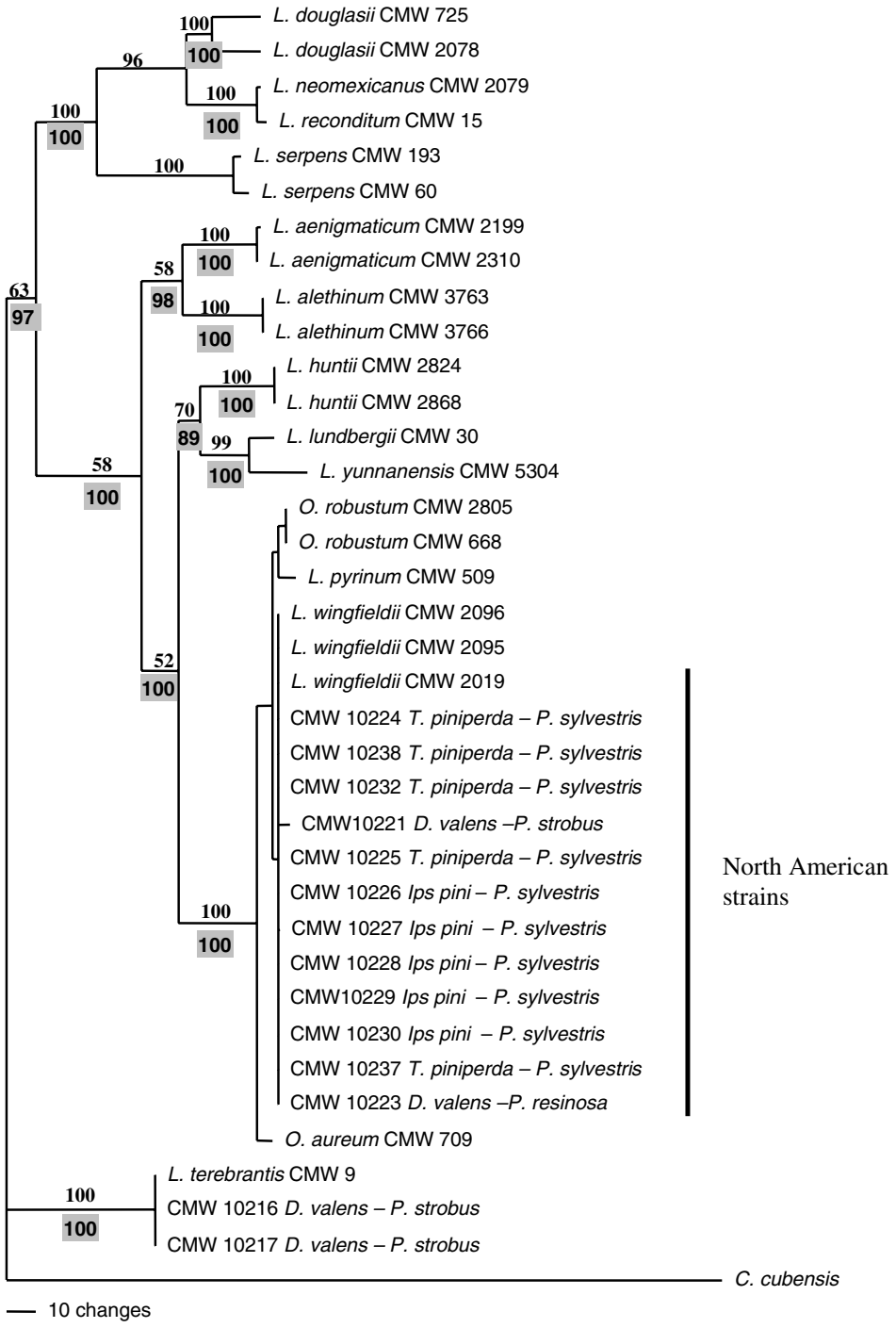


Fig. 1. One of the three most parsimonious trees of the combined ITS, β-tubulin and elongation 1-α gene data sets showing the relationship among different species of *Leptographium*. The strains from North America are grouped together with the authentic strains of *L. wingfieldii* from Europe. The shortest tree is 632 steps (CI = 0.839, RI = 0.897, HI = 0.161). *Cryphonectria cubensis* has been used as an outgroup. Bootstrap values are indicated on top of the branches and posterior probabilities for the nodes are indicated in boxes underneath branches.

Strains used for comparison included *Leptographium douglasii* (CMW725, AY553380[a], AY534928[b], AY536174[c]); *L. douglasii* (CMW2078, AY553381[a], AY534929[b], AY536175[c]); *L. neomexicanus* (CMW2079, AY553382[a], AY534930[b], AY536176[c]); *L. reconditum* (CMW15, AY553383[a], AY534931[b], AY536177[c]); *L. serpens* (CMW193, AY553387[a], AY534935[b], AY536181[c]); *L. serpens* (CMW60, AY553388[a], AY534936[b], AY536182[c]); *L. aenigmaticum* (CMW2199, AY553389[a], AY534937[b], AY536183[c]); *L. aenigmaticum* (CMW2310, AY553390[a], AY534938[b], AY536184[c]); *L. alethinum* (CMW3763, AY553391[a], AY534939[b], AY536185[c]); *L. alethinum* (CMW3766, AY553392[a], AY534940[b], AY536186[c]); *O. huntii* (CMW2824, AY553393[a], AY534941[b], AY536187[c]); *O. huntii* (CMW2868, AY553394[a], AY534942[b], AY536188[c]); *L. lundbergii* (CMW30, AY553395[a], AY534943[b], AY536189[c]); *O. robustum* (CMW2805, AY553396[a], AY534944[b], AY536190[c]); *O. robustum* (CMW668, AY553397[a], AY534945[b], AY536191[c]); *L. aureum* (CMW709, AY553413[a], AY534961[b], AY536207[c]); *L. pyrinum* (CMW509, AY553414[a], AY534962[b], AY536208[c]); *L. yunnanensis* (CMW5304, AY553415[a], AY534963[b], AY536209[c]); *C. cubensis* (C15, AY553416[a], AY534964[b], AY536210[c]).

infested with *Dendroctonus valens*, were all characterized by typical *Leptographium* conidiophores and slightly elongated penicilli. The conidia were highly variable in length and width and occasionally slightly clavate. This is similar to observations of the ex-type strain of *Leptographium wingfieldii* (CMW 2096) (Jacobs & Wingfield 2001) and those described by Morelet (1988) for this fungus. The conidiophores of these strains also had a slightly yellow colour, similar to those of the conidiophores of *L. wingfieldii* (Jacobs & Wingfield 2001) (Figs 2–7). Some strains from *P. resinosa* and *P. strobus* attacked by *D. valens* were similar to *L. wingfieldii* and the morphologically similar species, *L. terebrantis*.

Phylogenetic analyses

Amplification of the ITS2 and 28S region resulted in fragments of approximately 1000 base pairs (bp). The aligned data set consisted of 621 characters, including 441 constant characters, 49 parsimony informative characters and 131 uninformative characters. A heuristic search of the data set resulted in a single most parsimonious tree (MPT) of 230 steps (CI = 0.935, RI = 0.928, HI = 0.065, results not shown). Amplification of a part of the β-tubulin gene resulted in fragments of approximately 350–400 bp. The aligned data set consisted of 474 characters, including 188 constant, 29 parsimony uninformative and 60 informative characters. Two ambiguously aligned regions (187 bp) were identified and excluded from the analysis. These regions were replaced by weighted, coded characters (Lutzoni *et al*. 2000). Gaps were treated as a fifth base. A heuristic search of the data set resulted in 8 MPTs of 182 steps (CI = 0.802, RI = 0.925, HI = 0.198, results not shown). Amplification of a part of the EF 1-α gene resulted in fragments of approximately 900 bp. The aligned data set consisted of 928 characters, including 325 constant characters, 75 parsimony informative and 71 uninformative characters. Eight ambiguously aligned regions were identified (457 bp in total) and excluded from the analysis. These regions were replaced by weighted, coded characters (Lutzoni *et al*. 2000). Gaps were treated as a fifth base. A heuristic search of the data set resulted in 27 MPTs of 292 steps (CI = 0.856, RI = 0.884, HI = 0.144, results not shown).

The Templeton Nonparametric Wilcoxon Signed Ranked test showed no significant differences among the different data sets. The combined aligned data set consisted of 1373 characters, 997 of these were constant, 194 were parsimony uninformative and 192 were parsimony informative. A heuristic search of the combined data set resulted in three MPTs with the shortest tree 632 steps (CI = 0.839, RI = 0.897, HI = 0.161) (Fig. 1).

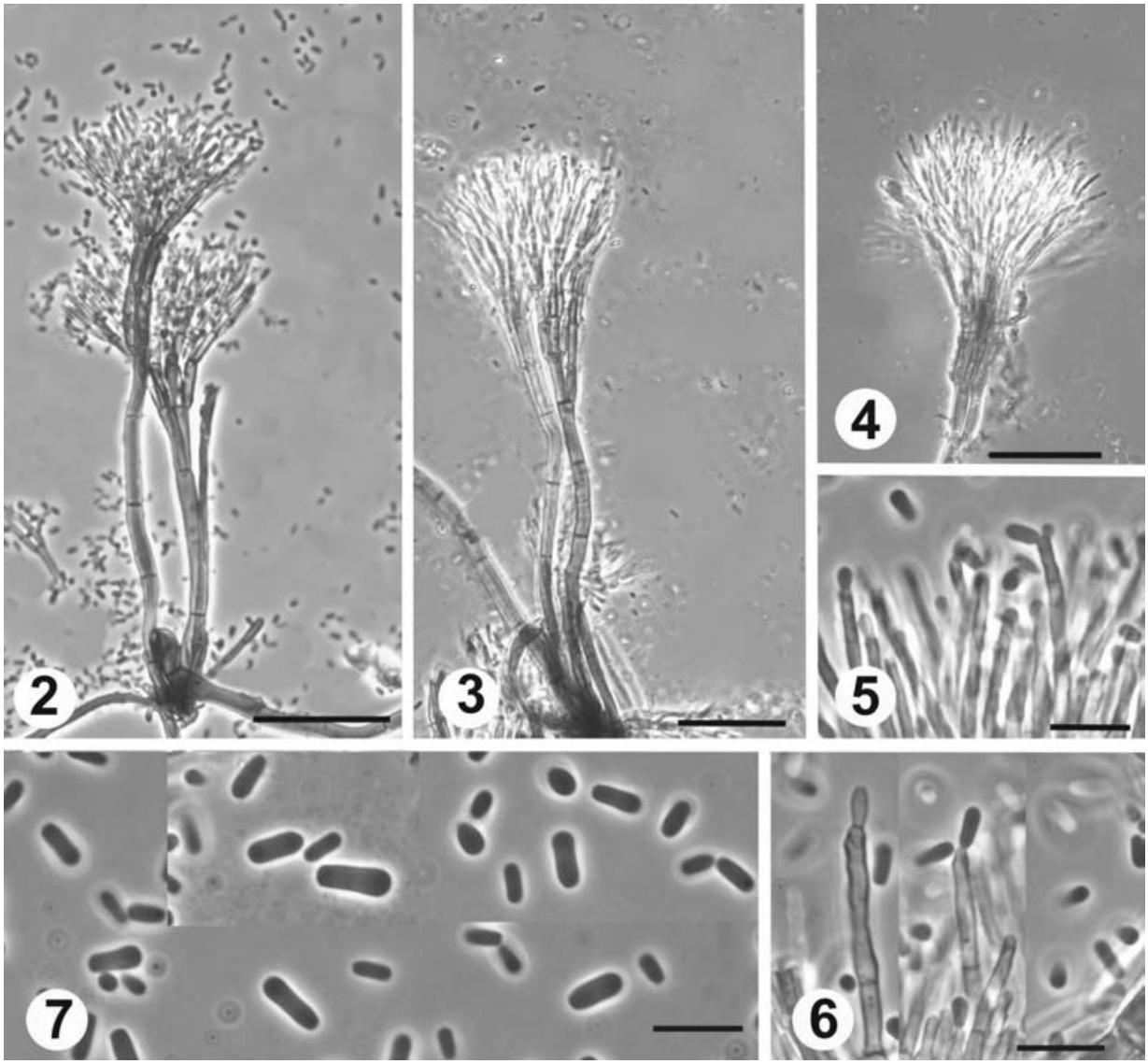
In all three data sets, the strains isolated from *P. sylvestris* attacked by *T. piniperda* and *I. pini* as well as strains isolated from pines attacked by *D. valens* grouped together with strains of *L. wingfieldii*, including the ex-holotype strain (CMW 2096). All strains identified as *L. wingfieldii* had identical ITS2, 28S, β-tubulin and EF 1-α sequences, except for CMW 10221 which had a single bp substitution in the EF 1-α set. Some strains isolated from the pines attacked by *D. valens* clustered together with *L. terebrantis*. This was also reflected in the trees resulting from the combined data set. In all cases, the clades were supported by high bootstrap values and the posterior probability of the nodes supported the bootstrap values (Fig. 1).

DISCUSSION

Fungal strains isolated from *Pinus sylvestris* infested by *Tomicus piniperda* in Michigan, and from *Ips pini* and *Dendroctonus valens* infested pines in Vermont, closely matched descriptions of *L. wingfieldii* (Morelet 1988, Jacobs & Wingfield 2001). They all had the slightly yellow conidiophores and variably sized, elongated oblong conidia that are characteristic for this species. This identification of *L. wingfieldii* was also supported by comparisons of sequence data for the ITS, β-tubulin and EF 1-α genes, in which the North American strains had identical sequences to the ex-type and other reference strains of the fungus. Together, these reports constitute the first definitive records of *L. wingfieldii* in North America.

The discovery of *L. wingfieldii* in North America is not surprising. The fungus is a common associate of *T. piniperda* in Europe and its introduction into North America with this insect might well have been expected. It is likely that the fungus was present from the time of the initial introduction of *T. piniperda* into the USA, and that the presence of the fungus was not noted until attempts were made to isolate it in this study. Presently, nothing is known regarding the actual or potential impact of *L. wingfieldii* on North American pines. In Europe, it has a relatively high pathogenicity (Lieutier *et al*. 1989, Solheim & Långström 1991) and it is likely to behave similarly in its new extended range. Furthermore, *T. piniperda* now infests hosts not previously associated with it in Europe and the outcome of these complex interactions is difficult to predict.

An important discovery is that *L. wingfieldii* is now associated with native North American bark beetles such as *I. pini* and *D. valens*. This is unlike the European situation where *L. wingfieldii* is exclusively associated with *T. piniperda*. Some *Leptographium* spp. are specific to their insect vectors, whereas others are associated with a diversity of bark beetles (Olchowecki & Reid 1974, Harrington & Cobb 1988, Jacobs & Wingfield 2001). Prior to this report, *L. wingfieldii* was only known from *T. piniperda*. The implications of its new association with native North American bark beetles and on new hosts are difficult to predict but deserve careful monitoring.



Figs 2–6. Morphological characters of *Leptographium wingfieldii* from North America (CMW 10225). **Figs 2–4.** Conidiophores with slightly elongated penicilli. Bar = 50 μm. **Figs 5–6.** Conidiogenous cells of *L. wingfieldii* showing percurrent proliferation of conidiogenous cells, and delayed secession of the conidia, a character typical of *Leptographium* spp. **Fig. 7.** Oblong conidia of *L. wingfieldii*, occasionally slightly elongated.

Some strains isolated from *Pinus* spp. attacked by *D. valens* in this study were identified as *Leptographium terebrantis* (Barras & Perry 1971, Jacobs & Wingfield 2001). This fungus is a well-known associate of *D. valens* in North America (Barras & Perry 1971, Wingfield 1983) and it is morphologically similar to *L. wingfieldii*. Both fungi have typical penicillate conidiophores, although those of *L. wingfieldii* are slightly more yellowish and elongated. Both these species have elongated conidia (Jacobs & Wingfield 2001). DNA sequence comparisons also showed that these fungi are closely related but that they can clearly be distinguished based on the gene regions selected. Previous DNA-based comparisons of *Leptographium* spp. have employed the ITS2 region and the 28S region of the ribosomal DNA operon (Jacobs, Wingfield & Wingfield 2001). In this study, we have also used both the β-tubulin and EF 1-α genes. β-tubulin sequences have previously been employed in species level phylogenetic studies in *Ophiostoma* (Jacobs & Kirisits 2003, Kim *et al*. 2003). This is the first time that sequences for the EF 1-α gene have been produced for *Leptographium* spp. and they are clearly useful for this purpose. Because of the morphological similarity of many *Leptographium* spp., reliance on DNA sequence data for identification of these fungi is growing.

Ecologically, *L. wingfieldii* and *L. terebrantis* are easily separated. In Europe, *L. wingfieldii* is consistently isolated in association with *T. piniperda* on *Pinus* spp. while *L. terebrantis* occurs in association with *D. terebrans* in North America (Barras & Perry 1971, Wingfield 1983). *Ophiostoma minus* is also associated with *T. piniperda* in Europe although there it is not as pathogenic as *L. wingfieldii* (Mathiesen 1951,

Mathiesen-Käärik 1953, Lieutier *et al*. 1989, Solheim & Långström 1991, Solheim *et al*. 1993, Långström *et al*. 1993). Identification and monitoring of *L. wingfieldii* are likely to become more difficult in North America, where this fungus has already established an overlapping niche with those of *L. terebrantis* and *O. minus*.

A fungus tentatively identified as *L. wingfieldii* was collected in 1993 from Niagara Falls, Ontario area, near the border of Ontario, Canada and New York State, USA, when *T. piniperda* was first encountered (D. Bright, K. A. Seifert & M. J. Wingfield, unpubl. data). *T. piniperda* was reported from Ontario in 1993, and has subsequently spread over southern Ontario and parts of Quebec (Bright 1996, Haack 1996, Canadian Food Inspection Survey 2001, Morgan *et al*. 2002). The original Ontario strain sporulates poorly but the herbarium specimen is in good condition. DNA comparisons confirm its identity and this isolate can now be recognized as the first of the species collected in North America. Furthermore, its collection closer to the time of the first appearance of *T. piniperda* is evidence that *L. wingfieldii* entered North America at the time of introduction of the insect. Unfortunately, no fungal isolations were made from the first detections of *T. piniperda* in USA in the early 1990s, and these specimens should be examined for the presence of *L. wingfieldii* in the future.

T. piniperda is native in Europe and throughout south-east Asia, including China. In Yunnan Province, the insect known as *T. piniperda* apparently does not carry *L. wingfieldii* but is the vector for *L. yunnanensis* (Zhou *et al*. 1998, 1999, 2001). *T. piniperda* apparently also occurs in other parts of China, but the fungi associated with this insect in these areas have not been identified.

The pathogenicity of *L. wingfieldii* to native pines in North America should be assessed to consider its potential importance in North America. This fungus may enhance the importance of bark beetles previously not associated with fungi with high levels of pathogenicity. In time, *L. wingfieldii* could replace other North American blue stain fungi that are less aggressive or it might equally be out-competed by fungi that are better adapted to the environment. More detailed studies should also be undertaken to monitor the occurrence of *Ophiostoma* and *Leptographium* spp. associated with *T. piniperda* and other insects that overlap its newfound niche.

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