Fungi vectored by the introduced bark beetle *Tomicus piniperda* in Ontario, Canada, and comments on the taxonomy of *Leptographium lundbergii*, *Leptographium terebrantis*, *Leptographium truncatum*, and *Leptographium wingfieldii*

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Abstract: Fungi isolated from *Tomicus piniperda* (L.) galleries in infected trap logs, standing trees, and directly from insects were identified using morphological features and molecular data obtained from the mitochondrial and nuclear DNA region. Identified strains represented *Leptographium wingfieldii* Morelet, *Leptographium procerum* (Kendr.) Wingf., *Leptographium lundbergii* Lag. & Melin sensu Jacobs & Wingfield, *Ophiostoma ips* (Rumb.) Nannf., *Ophiostoma minus* (Hedg.) H. & P. Syd., and *Sphaeropsis sapinea* sensu lato. *Leptographium wingfieldii* is believed to be a potentially pathogenic introduced fungus, but sequence data suggest a possible connection between it and the teleomorph of *Ophiostoma aureum* (Robinson-Jeffrey & Davids.) T.C. Harrington (reported from British Columbia and the western United States). Our data also show that the ex-type culture of *Leptographium terebrantis* Barras & Perry, a species very similar morphologically to *L. wingfieldii*, also grouped with *L. wingfieldii*. We also identified strains of *Leptographium truncatum* (Wingf. & Marasas) Wingf.; this species has been synonymized with *L. lundbergii*, but our data indicate that these are distinct species, and therefore, the name *L. truncatum* should be reinstated. We also report the extended presence of *L. procerum* in Ontario. Previously viewed as a "southern" species frequently associated with pine-root decline diseases, it has been infrequently reported from New York state and but once each from Ontario and Quebec.

Key words: bark beetles, blue-stain fungi, *Leptographium*, *Ophiostoma*, ITS regions.

Résumé : Les auteurs ont isolé les champignons qui se retrouvent dans les galeries du *Tomicus piniperda* (L.), formées dans des tronçons pièges et des arbres debout, ainsi que directement à partir d'insectes; ils ont identifié ces champignons en utilisant des caractéristiques morphologiques, et des données moléculaires obtenues de régions de l'ADN nucléique et mitochondrial. Les espèces identifiées regroupent les *Leptographium wingfieldii* Morelet, *Leptographium procerum* (Kendr.) Wingf., *Leptographium lundbergii* Lag. & Melin sensu Jacobs & Wingfield, *Ophiostoma ips* (Rumb.) Nannf., *Ophiostoma minus* (Hedg.) H. & P. Syd et le *Sphaeropsis sapinea* sensu lato. On croit que le *L. wingfieldii* est un champignon introduit potentiellement pathogène, mais les données de séquençage suggèrent une relation possible entre cette entité et le téléomorphe de l'*Ophiostoma aureum* (Robinson-Jeffrey & David) T.C. Harrington (mentionné en Colombie Canadienne et dans l'ouest des USA). Les données obtenues à partir de cultures de l'ex-type du *Leptographium terebantis* Barras & Perry, une espèce morphologiquement très semblable au *L. wingfieldii*, montrent que cette entité se regroupe également avec le *L. wingfieldii*. Les auteurs ont de plus identifié des souches du *Leptographium truncatum* (Wingf. & Marasas) Wingf.; on a déjà fait de cette espèce un synonyme du *L. lundbergii*, mais les données ici présentées indiquent qu'il s'agit d'espèces distinctes et, conséquemment, le nom de *L. truncatum* devrait être rétabli. Les auteurs rapportent également une présence extensive du *L. procerum* en Ontario. Auparavant considérée comme une espèce du sud, souvent associée aux maladies du dépérissement racinaire chez les pins, cette entité a été occasionnellement mentionnée dans l'état de New York, et une fois chacune pour les provinces d'Ontario et de Québec.

Received 21 February 2005. Published on the NRC Research Press Web site at http://canjbot.nrc.ca on 8 December 2005.

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 doi: 10.1139/b05-095

Mots clés : scolytes, champignons du bleuissement, *Leptographium*, *Ophiostoma*, régions de l'ITS.

[Traduit par la Rédaction]

Introduction

Tomicus piniperda (L.), the pine shoot beetle (PSB), is a widely distributed Palearctic species that has been introduced repeatedly into North America (Carter et al. 1996; Kerdelhué et al. 2002; Siegert and McCullough 2003) before becoming firmly established in the United States by 2003 (National Pest Information System, http://www.ceris.purdue.edu/napis/pests/psb/imap/psbt03.html). First detected in Ontario, Canada, in 1993, then Quebec in 1998, by 2002 it was established in 29 Ontario counties and 19 Quebec counties. Initially in Ontario, shoot damage was confined largely to stressed and weakened Scots pine (*Pinus sylvestris* L.), but it is now reported causing widely distributed damage on eastern white pine (*Pinus strobus* L.), red pine (*Pinus resinosa* Ait.), and jack pine (*Pinus banksiana* Lamb.) (Morgan et al. 2004). These authors studied selected sites composed of jack, red, and Scots pines in various compositions in southern Ontario and found that PSB attacked these tree species, but not with equal ease, and that the PSB is potentially manageable by silvicultural practices. This suggests fungi vectored by the PSB might be distributed to a variety of host species.

In Scandinavia, the beetle is considered a nonaggressive pest of Scots pine that can cause extensive economic loss because of shoot feeding (Långström 1983; Långström and Hellqvist 1990; Lieutier 1991; Långström et al. 1992; Krokene et al. 2000), but resin produced in beetle-induced lesions usually "pitches them out" of healthy trees. Although an efficient defense mechanism even at high attack densities, this is often significantly negated if tree vigour has been reduced prior to attack (Långström et al. 1992; Annila et al. 1999). In 1988 in southern Britain more than 17% of the spring-emergent overwintered adult beetles carried strains of *Leptographium* spp., as did over half of the emergent June–July adult brood (Gibbs and Innman 1991). These authors found that 95% of their isolates were *Leptographium wingfieldii* M. Morelet, plus strains of *Leptographium lundbergii* Lagerb. & Melin, *Leptographium huntii* M.J. Wingf. [teleomorph *Ophiostoma huntii* (Robinson-Jeffrey) de Hoog & Scheffer], *Leptographium procerum* (W.B. Kendr.) M.J. Wingf., and other unidentified entities.

While many *Leptographium* spp. are true anamorphic fungi associated with bark-beetle vectors, others have teleomorphic states in the genus *Ophiostoma* (Jacobs and Wingfield 2001), and often are either blue-staining or primary pathogens. Thus introduction of the beetle into North America might also have introduced exotic fungal species with important destructive potential.

In 1989, Piou and Lieuter reported finding extensive PSB-associated Scots pine mortality in central France. While this was atypical to that noted normally in Scandinavia, Lieutier et al. (1989*a*, 1989*b*) and Piou and Lieuter (1989) found two other pathogens, *L. wingfieldii* and *Ophiostoma minus* (Hedgc.) Syd. & P. Syd., associated with the beetle that might have contributed to the unusual mortality rate. Solheim and Långström (1991), Långström et al. (1993), and Solheim et al. (1993) studied a variety of factors related to possible interactions between PSB attacks and tree mortality. Among other conclusions reached was that the PSB did introduce fungi into host trees, and that one of these, *L. wingfieldii*, can kill healthy trees.

Other fungi reported to be associated with the PSB are *L. lundbergii* (Lagerberg et al. 1927; Wingfield and Marasas 1983; Kaneko and Harrington 1990), *L. procerum* (Lackner and Alexander 1982; Wingfield 1986; Morrison and Hunt 1988; Otrosina et al. 1997, 1999, 2002), and *O. minus* (Nelson 1934; Mathre 1964; Basham 1970; Paine and Stephen 1987; Otrosina et al. 2002). However, some reports of *L. lundbergii* being present in Japan and North America stem from the fact that Harrington (1988) and Kaneko and Harrington (1990) reported the occurrence of *Verticicladiella truncata* M.J. Wingf. and Marasas (Wingfield and Marasas 1983) in these countries. This species was later reduced to synonomy with *L. lundbergii* (Wingfield and Gibbs 1991; Strydom et al.1997), and this has caused confusion.

This study began in April 2000 because the rapid spread of *T. piniperda* raised two concerns: first, was it carrying exotic pathogens such as *L. wingfieldii*; second, might it also become an additional vector of indigenous pathogens? Standard morphological techniques and molecular criteria were employed to establish precisely which *Leptographium* spp., if any, were associated with PSB in Ontario because conventional morphological characters are often quite variable in species of this genus (Zambino and Harrington 1992; Wingfield 1993; Hausner et al. 2000*a*). However, as the sole intention was to determine which fungal species were associated with the PSB in the sampled sites, no attempt was made to analyze species frequencies or niche relatedness between sites, but to merely report occurrence.

Three different genome regions were used to resolve relationships amongst strains identified morphologically as *L. wingfieldii*. One data set comprised partial sequences of a group I intron inserted in the U11 region of the mitochondrial ribosomal large subunit gene (mt-*rnl*) (Burke and RajBhandary 1982; Cummings et al. 1989; Hausner et al. 1999*a*; Hausner 2003). A second data set was generated by analysing the rDNA spacer (IGS) between the nuclear ribosomal RNA genes; this region has been of great value in species identification and resolving intraspecific relationships (Hausner et al. 2000*b*). Therefore, the polymerase chain reaction (PCR) was used to amplify the IGS region of 21 *Leptographium* strains, and the resulting PCR products were analyzed for restriction fragment length polymorphisms (RFLPs).

The rDNA internal transcribed spacer regions (including the 3′ end of *rns*, ITS1, the 5.8S gene, ITS2, and *rnl* 5′ end) was also sequenced to serve as a third data set. ITS sequences have been applied to evolutionary comparisons etc. in the fungi (Guarro et al. 1999), but few complete ITS regions of *Leptographium* spp. are currently available due to techni-

cal difficulties (Jacobs et al. 2001). However, here we report ITS region sequences of 47 putative *Leptographium* strains including strains we identified morphologically as *L. procerum* and *L. lundbergii* (fide Jacobs and Wingfield 2001).

We hoped the phylogenetic analysis would confirm our morphological placements because we encountered significant morphological variation in presumed species populations. We also included ex-holotype strains for *Leptographium truncatum*, *Leptographium terebrantis*, *Ophiostoma aureum*, and ex-neotype strains for *L. lundbergii* and *Ophiostoma penicillatum* (Grosm.) Siemaszko in our analysis of ITS sequences because of issues that arose during this study.

Material and Methods

Sampling methods for blue-stain fungi

Woody tissue collections and fungal isolations

During the spring and summer of the year 2000, recently dead or dying Scots pines having either PSB galleries or beetles present were sampled at six locations throughout southern Ontario (see Table 1). Selected trees were felled, sectioned using a chain saw, and blue-stained sections were refrigerated at –10 °C until isolations were made.

During the late winter of 2001, 51-cm-long bolts were cut from healthy Scots pine growing in Kirkwood Provincial Forest just north of Thessalon, approximately 83 km east of Sault Ste. Marie, Ontario on provincial highway 17; well north of the then known distribution of the PSB in Ontario.

Cut bolts, rough-barked and typically suitable for *Tomicus* habitation, were 20–25 cm in diameter. Ends were sealed with paraffin wax to help prevent dessication, and eight bolts were placed in each collection site late in winter before the spring mating-flight period of adult beetles. Eight similar control bolts were placed in an insect-rearing room set at 20 °C and 80% relative humidity (RH) at the Great Lakes Forest Research Centre (GLFC), Canadian Forest Service, Sault Ste. Marie.

After the spring beetle-mating flight, at one-month approximate intervals, two logs were collected from each site. These, along with a control bolt, were wrapped in plastic and frozen until isolations were made.

In late winter of 2002, again before the beetle mating-flight period, two apparently healthy Scots pines were felled and left on the ground in each previously sampled plantations. Two months after the beetle-mating flight, two 51-cm bolts were cut from these felled trees at one-month intervals and were frozen. Next, eight control bolts were cut from two healthy Scots pine in Kirkwood Forest; two of these were frozen with the monthly collected bolts, but six were placed in an insect-rearing room. Bolts always had rough bark and were from lower tree ends.

Isolation substrates were the following: (1) MEA–YE medium (MEA; 20 g malt extract, Difco, Detroit, Michigan; 20 g bacteriological agar GIBCO BRL, Paisley, Scotland; and 1 g yeast extract, Gibco; all per litre); (2) MEA–YE containing 1 g/L of penicillin G and streptomycin sulphate (Sigma Chemicals, St. Louis, Missouri); and (3) 2–3 cm diameter wood discs, 0.4 cm thick, cut from living Scots pine branches. The latter were submersed in water, autoclaved at 121 °C for 1 h twice in a 24 h period, and placed aseptically into sterile plastic Petri dishes containing MEA–YE agar. The discs served as the growth medium.

Bolts collected in 2000 were debarked, and the exposed surface was examined for fungal fruiting structures. When present, spore masses were transferred to a selected substrate and dark incubated at 20 °C. From exposed surfaces of wood bolts dissected with either an electric wood-splitter, band saw, or hand axe, small subsurface chips were excised aseptically, plated onto both MEA–YE and MEA–YE plus antibiotics agars and incubated as above.

When uniform colonies grew, these were transferred onto sterile Scots pine discs, incubated as above, and examined regularly. Again, when fruiting occurred, sub-cultures were made onto MEA–YE plates from individual spore masses. After 1 week, stock tubes were made, and both culture plates and the wood discs were prepared and stored for future reference.

Isolations from the beetles

Lindgren traps were used to collect spring emerging beetles at Drysdales' Tree Farm and both Conestogo Lake Area locations. In 2001, trap baits were 95% α-pinene (99% pure, Phero Tech Inc., Delta, British Columbia), released from two 15 mL vials at a rate of 300 mg/d (a day is a 24 h period). In 2002, the α-pinene was released concurrently with trans-verbenol (95% pure, 3.2% cis), myrtenol (94% pure), and nonanal (94% pure), from individual bubble caps at 1.5, 0.75, and 13 mg/d, respectively (Phero Tech).

Four protocols were employed to isolate fungi: (1) beetles were allowed to walk on the surfaces of both types of medium plates; (2) crushed beetles were smeared on plates; (3) beetles were washed in sterile distilled water, and the water was used in a dilution series, with 1.0 mL from each dilution being spread over the surfaces of individual media (1 or 2) plates; and (4) washed beetles were crushed on media plates.

All plates were dark incubated at 20 °C for several days, examined regularly and strains of interest transferred to MEA–YE plates that were incubated as above. These were then transferred to wood discs, grown, identified, and stocked. All the strains studied are listed in Table 2, as are the sources of strains used for comparative purposes. The latter included authentic strains (including ex-holotype, ex-paratype, and ex-neotype strains) for some of the key *Leptographium* species of importance in forest pathology. All strains are maintained as live cultures on slants of MEA–YE.

Maintenance and culturing of fungal strains

We cultured strains on wood discs, a technique used to better determine the potential range of variation in morphological features that might occur on natural substrates, and on MEA–YE agar (Hausner et al. 2003*a*).

Morphological structures used to identify strains were mounted in 85% lactic acid (Fisher Scientific, Fair Lawn, New Jersey) on slides; these were then placed on a slide warmer set 40 °C for at least 45 h before ringing with nail varnish. When required, a minimum of 50 spores were measured, and 20 of each of the other relevant morphological structures from both culture substrates and both ranges and averages (means) were computed.

Table 1. The *Tomicus*–blue-stain study trap-log collection locations

Site	Location	Global position satellite (GPS = UTS)
1	Conestogo Lake Conservation Area, SE side, Maryborough Township (Twp), near Listowel, Ontario	17 523145 4836551
2	Conestogo Lake Conservation Area, NW side, Maryborough Twp., near Listowel, Ontario	17 0517542 4840468
3	Valens Conservation Authority, Flamborough Twp., near Cambridge, Ontario	17 0568741 4804225
4	Drysdale's Tree Farm. Essa Twp., near Barrie, Ontario	17 595109 4899086
5	Near Terra Nova, Mulmur Twp., Ontario	17 571438 4898699
6	Near Bracebridge, Draper Twp., Ontario	17 0634139 4992834

Amplification of a mtDNA *rnl* U11 intron, nuclear rDNA ITS, and IGS regions, and DNA sequencing

For DNA purification and extraction protocols see Hausner et al. (1992*a*). Whole cell DNA served as the template for amplification of DNA fragments of interest using the Invitrogen-BRL PCR System (Buffer and *Taq* polymerase, Invitrogen). Primers SSUZ and LSU4 (Hausner et al.1993*a*) were used to amplify ITS regions and mtDNA *rnl* U11 intron segments were amplified with primers IP1 and IP2 (Bell et al. 1996). The PCR primer sequences, amplification conditions, sizes of the expected PCR products, and preparation of sequencing templates for ITS and mtDNA *rnl* U11 intron fragments have been described previously (Hausner and Wang 2005; Gibb and Hausner 2005, respectively). Double-stranded PCR products were sequenced using the cycle-sequencing protocols performed according to the manufacturers' recommendations (Perkin Elmer Applied Biosystems, Foster City, Calif.), and automated fluorescent DNA sequence analysis was performed using an ABI Prism 310 Genetic Analyzer system (PEAB at the University of Calgary, DNA sequencing facility, Calgary, Alta.). The amplification of the nuclear ribosomal IGS region with primers E and F2 (White et al. 1990; Kim et al. 1992) and the protocols used for RFLP analysis have been described in Hausner et al. (2000*b*). Endonuclease digestions of the IGS PCR products with *Alu*I, *Cfo*I, *Hinf*I, *Hae*III, *Mbo*I, *Mse*I, *Msp*I, *Rsa*I, and *Taq*I were performed using PCR buffer-compatible enzymes (Invitrogen Life Technologies) following the recommendations of the manufacturer, except that 5 units of restriction enzyme were added directly to 5 μL of PCR mix after the PCR reaction was completed. Electrophoresis was carried out in submarine gels at 100 V for 1.5 h in TBE buffer (89 mmol/L TrisHCl, 89 mmol/L boric acid, 10 mmol/L EDTA, pH 8.0). The gels employed were 20 cm × 10 cm × 0.5 cm horizontal 1.0% agarose (Invitrogen) for uncut PCR products with some restriction fragments being resolved on 1.8% agarose, e.g., *Alu*I, *Cfo*I, *Hae*III, *Rsa*I, and *Taq*I digests. However, 3.0% Metaphor agarose (BioWhittaker Molecular Applications, Rockland, Me.) was used to resolve fragments generated when PCR products were digested with *Hinf*I, *Mbo*I, *Mse*I, and *Msp*I. The 1 kb plus and 100 bp (Invitrogen) ladders were used as molecular weight standards. Agarose gels were stained for 25 min with ethidium bromide (0.5 μg/mL in TBE buffer) followed by 20 min of destaining in water. Then, under UV light, the images were digitized using the BioRad Gel Documentation System (BioRad Gel Doc 1000; BioRad Laboratories (Canada) Ltd., Mississauga, Ontario).

Analyses of DNA sequence data and restriction fragment length polymorphisms

ITS and mt-*rnl* U11 DNA sequences were aligned with CLUSTAL-X (Thompson et al. 1997) and when appropriate, modified with an alignment editor program (GeneDoc v2.5.010; Nicholas et al. 1997). Programs contained within PHYLIP (version 3.6a; Felsenstein 2002) were used for phylogenetic analysis of the aligned mt-*rnl* U11 data set. The aligned data set contained partial sequences covering 425 sites that comprise the 5′ end of the U11 intron in 25 tested strains (GenBank accession Nos. listed in Table 2). Divergence (or distance) between two sequences was calculated by DNADIST (setting F84). A tree was constructed with the NEIGHBOR program (UPGMA option), and evaluated using the bootstrap procedure (1000 replicates) in PHYLIP.

The IGS rDNA restriction patterns were converted into a binary data set (0 = absence of band; 1 = presence of a band), and from pairwise comparisons similarity coefficients (*S* values) were calculated (Nei and Li 1979) and the *S* values were compiled in a genetic distance matrix (Swofford and Olsen 1990). The latter was used to construct dendrograms with the NEIGHBOR (NJ option) program (PHYLIP).

The aligned ITS data set contained 51 sequences covering 672 positions (see Table 2 for GenBank accession Nos.) and included ITS sequences for *Ophiostoma ips* (GenBank AF198244), *Ophiostoma cucullatum* (AF198246), and *Ophiostoma piceae* (AF081131) to serve as outgroups. Programs contained within PHYLIP (Felsenstein 2002) and Tree-Puzzle (Schmidt et al. 2002) were applied to resolve phylogenetic relationships among the tested sequences. The data set was analyzed with DNAPARS (maximum parsimony) and DNADIST (F84 setting). From the latter, the distance matrix generated was used in the NEIGHBOR program (NJ setting) for inferring a phylogenetic tree. The phylogenetic estimates were evaluated using the bootstrap procedure (SEQBOOT 1000 replicates; and CONSENSE) in PHYLIP. The ITS data was also analyzed with the Tree-Puzzle program (maximum likelihood (ML) phylogenetic analysis using quartets and parallel computing (Schmidt et al. 2002)). The settings for the quartet puzzling algorithm were as follows: 10 000 puzzling steps, transition–transversion parameter estimated from data set, HKY evolutionary model (Hasegawa et al. 1985).

The phylogenetic trees and dendrogram presented were drawn with the TreeView program (Page 1996) using the PHYLIP tree outfiles, and annotations were added to the figures with the aid of Corel Draw (Corel Corporation and Corel Corporation Limited).

Table 2. List of strains used, their sources, and GenBank accession numbers for the molecular data obtained for this study.

Isolate WIN(M) No.	Collector or supplier No.	Substrate	Collector and country of origin	Identifier (notes)	GenBank accession No. *rnl* U11	GenBank accession No. ITS region
Leptographium lundbergii						
WIN(M) 68	NFRI 60-25	*Pinus sylvestris*	A.M.-K., Sweden	*L. lundbergii*		AY935584
WIN(M) 69	NFRI 1502/1	*P. sylvestris*	R.-H., Norway	*L. lundbergii*		AY935583
WIN(M) 967	NFRI 69-148	*P. sylvestris*	R.-H., Norway	*L. lundbergii*		AY935588
WIN(M) 1115	CBS 352.29	N/A	Melin, Sweden*	*L. lundbergii* neotype[†]	AY275156	AY935585
WIN(M) 1129	DAOM 60397	Wood	Not available, Sweden	*L. lundbergii*		AY935587
WIN(M) 1131	NFRI 89-1040/1/3	*P. sylvestris*	Solheim, Sweden	As *L. wingfieldii*; re-id. as *L. lundbergii* J.R	AY275155	AY935586
WIN(M) 1194	DSMZ 5010	*Picea* sp.	Butin, Germany	*L. lundbergii*		AY935589
WIN(M) 1197	DAOM 63692	*Pinus* sp.	A.M.-K., Sweden	*L. lundbergii*		AY935590
WIN(M) 1438	CBS 352.29	Repeat sending	See WIN(M) 1115			(=AY935585)
Leptographium truncatum						
WIN(M) 174	NFRI 1813/1	N/A	R.-H., Norway	*Leptographium* sp.		AY935591
WIN(M) 254	Forintek C34	N/A	Lagerberg, Sweden	*L. lundbergii*		AY935595
WIN(M) 660	NFRI 59-7/3	*P. sylvestris*	N/A, Norway	*Leptographium* sp.		AY935601
WIN(M) 1028	J.R. 88-449	*P. radiata*	J.R., New Zealand	*Leptographium* sp.		AY935592
WIN(M) 1029	J.R. 88-324	*Pinus* sp.	J.R., New Zealand	*Leptographium* sp.		AY935593
WIN(M) 1128	DAOM 60396	N/A	Lagerberg, Sweden	*L. lundbergii*		AY935594
WIN(M) 1246	TOM 74.29	*P. sylvestris*	Davis, Canada (Ontario)	*L. lundbergii*		AY935581
WIN(M) 1274	TOM 86.30	*P. sylvestris*	Davis, Canada (Ontario)	*Leptographium* (?) sp.		AY935582
WIN(M) 1434	CBS 647.89	*P. sylvestris*	M. Morelet, France	*L. truncatum*		AY935627
WIN(M) 1435	CBS 929.85	*Pinus taeda*	M.J. Wingfield, South Africa	*L. truncatum* ex-holotype		AY935626
NZFS 169D			M. Dick, New Zealand	*L. lundbergii*		AY935625
Leptographium procerum						
WIN(M) 33	NFRI 59-84/2	Timber wood	N/A, Norway	*Phialocephala phycomyces;* re-id. as *L. procerum* by Harrington (1986)	AY275141	AY935618
WIN(M) 796	UAMH 9724	*Pinus nigra*	J.R., New Zealand	*Leptographium* sp.	AY275140	AY935621
WIN(M) 1199	DAOM 33940	*P. strobus*	S.N. Linzon, Canada	*L. procerum* id. S.J. Hughes		AY935613
WIN(M) 1210	TOM 62.30	*P. sylvestris*	Davis, Canada (Ontario)	*L. procerum* id. J.R.		AY935616
WIN(M) 1211	TOM 55.35	*P. sylvestris*	Davis, Canada (Ontario)	*L. procerum* id. J.R.	AY275159	(=AY935616)
WIN(M) 1244	TOM 73.12	*P. sylvestris*	Davis, Canada (Ontario)	*L. procerum* id. J.R.		AY935615
WIN(M) 1250	TOM 76.8	*Tomicus piniperda*	Davis, Canada (Ontario)	*L. procerum* id. J.R.		AY935614
WIN(M) 1254	TOM 76.36	*T. piniperda*	Davis, Canada (Ontario)	*L. procerum* id. J.R.		AY935617
WIN(M) 1272	TOM 86.19	*P. sylvestris*	Davis, Canada (Ontario)	*L. procerum* id. J.R.		AY935619
WIN(M) 1375	J.R. 88-409A	*Pinus* sp.	J.R., New Zealand	*L. procerum* id. J.R.		AY935620
Leptographium terebrantis						
WIN(M) 468	UAMH 9722	*Pinus contorta*	J.R., Canada (British Columbia)	*L. terebrantis* id. J.R.	AY275154	AY935605
WIN(M) 662	UAMH 9690	*P. contorta*	J.R., Canada (British Columbia)	*L. terebrantis* id. J.R.		AY935607
WIN(M) 1183	CBS 337.70	pupal chamber	Barras, USA (Louisiana)	*L. terebrantis* ex-holotype	AY275143	AY935609
WIN(M) 1184	CBS 298.85		M.J.W., USA	*L. terebrantis* id. M.J.W.		AY935598

Table 2. (*concluded*).

Isolate WIN(M) No.	Collector or supplier No.	Substrate	Collector and country of origin	Identifier (notes)	GenBank accession No.	
					rnl U11	ITS region
WIN(M) 1185	CBS 408.61	Gymnosperm, wood	Weitz, Germany	*Scopularia corsicana*, by B. Schol-Schwarz re-id. *L. terebrantis* W. Gams		AY935597
Leptographium wagneri						
WIN(905)	ATCC 58579	*Pinus edulis* Engelm.	R. James, U.S.A.	*Ceratocystis wagneri* id. Harrington T.C		AY935596
Leptographium wingfieldii						
WIN(M) 1118	CBS 645.89	*T. piniperda*	Morelet, France	*L. wingfieldii* ex-holotype	AY275151	AY935603
WIN(M) 1123	MCC 125	*Pinus densiflora*	Masuya, Japan	*L. wingfieldii* id. M. Masuya	AY275150	AY935608
WIN(M) 1124	MCC 130	*P. densiflora*	Masuya, Japan	*L. wingfieldii* id. M. Masuya	AY275147	AY935612
WIN(M) 1126	MCC 349	*T. piniperda*	Masuya, Japan	*L. wingfieldii* id. M. Masuya	AY275149	AY935610
WIN(M) 1130	NFRI 88-369/11	*P. sylvestris*	Solheim, Sweden	*L. wingfieldii* id. H. Solheim	AY275157	
WIN(M) 1181	CBS 648.89	*Pinus brutia*	Mich.-Ska., Greece	*L. wingfieldii* ex-paratype i.d. Morelet	AY275142	AY935611
WIN(M) 1182	CBS 345.90	*T. piniperda*	UK	*L. wingfieldii* id. M.J.W.	AY275142	
***L. wingfieldii* isolates from Ontario**						
WIN(M) 1100	TOM 1.3	*P. sylvestris*	Davis, Canada (Ontario)	*L. wingfieldii* id. J.R.	AY275146	
WIN(M) 1103	TOM 10.2	*P. sylvestris* (trap log)	Davis, Canada (Ontario)	*L. wingfieldii* id. J.R.	AY275148	AY935599
WIN(M) 1120	TOM 9.4	*P. sylvestris*	Davis, Canada (Ontario)	*L. wingfieldii* id. J.R.	AY275145	
WIN(M) 1121	TOM 11.5	*P. sylvestris*	Davis, Canada (Ontario)	*L. wingfieldii* id. J.R.	AY275152	AY935602
WIN(M) 1192	TOM 5.1	*P. sylvestris* (trap log)	Davis, Canada (Ontario)	*L. wingfieldii* id. J.R.	AY275153	AY935604
WIN(M) 1209	TOM 59.21	*P. sylvestris* (trap log)	Davis, Canada (Ontario)	*L. wingfieldii* id. J.R.	AY275158	AY935600
***Leptographium* sp.**						
WIN(M) 1376	J.R. 88-194A	*P. austriaca* Hoess	J.R., New Zealand	*Leptographium* sp.		AY935622
Ophiostoma aureum						
CBS 438.69 (ex-T)	WIN(M) 809	*Pinus contorta*	R.R.-J. & R.W.D, Canada (Britsh Columbia)	*O. aureum* ex-holotype id. R. Robinson-Jeffrey	AY275139	AY935606
Ophiostoma penicillatum						
WIN(M) 27	NFRI 60-21	*Picea abies*	A.M.-K, Sweden	*O. penicillatum* neotype (H. Solheim)	AY275138	AY935623
WIN(M) 544	DAOM63691	*P. abies*	A.M.-K., Sweden	*O. penicillatum* id. Kendrick		AY935624
Ophiostoma piceae						
WIN(M) 909	CBS 108.21	N/A	Germany	ex-holotype	AY275135	
Ophiostoma novo-ulmi						
WIN(M) 900		*Ulmus americana* L.	J.R., Canada (Manitoba)	*O. novo-ulmi* subsp. *americana* id. JR	AY275136	
WIN(M) 904		*Ulmus americana*	J.R., Canada (Manitoba)	*O. novo-ulmi* subsp. *americana* id. JR	AY275137	

Note: A.M.-K., A.M.-Käärik; M.J.W., M.J. Wingfield; J.R., J. Reid; R.R.-J. & R.W.D, R. Robinson-Jeffrey and R.W. Davidson; R.H., Finn Roll-Hansen; Mich.-Ska., Michalopoulos-Skaramoutsos; CBS, Centraal Bureau voor Schimmelcultures, Utrecht, Netherlands; WIN(M), University of Manitoba, Microbiology/Botany, the author's (J.R.) own collection; UAMH, University of Alberta Microfungus Collection & Herbarium; Devonian Botanic Garden, Edmonton, Alberta, Canada, T6G 2E1; ATCC, American Type Culture Collection, Rockville, Maryland, USA; Forintek, Forintek Canada Corp., 319, rue Franquet, Sainte-Foy, Québec G1P 4R4; NFRI, Norwegian Forest Research Institute, AS, Norway; DAOM, Cereal and Oilseeds Research, Agriculture & Agri-Food Canada, Ottawa, Ontario, Canada; MCC, culture collection of H. Masuya; TOM, isolation designation, Canadian Forest Service, Great Lakes Forestry Centre, 1219 Queen St., Sault Ste. Marie, Ontario, P6A 5M7; NZFS, New Zealand Forest Service.

*Melin is credited as the collector, but this might be in error.

†Recently, Jacobs et al. (2005) addressed the problem of the typification of *L. lundbergii* and the synonymization of *L. truncatum* with it. They abandoned CBS 352.29 and retypified *L. lundbergii* with NFRI 60-25 (see WIN(M) 68). They also separated *L. truncatum* from *L. lundbergii*.

Results and discussion

Fungi associated with *T. piniperda*

We obtained and tentatively identified 384 isolates (Table 3). Of these, 80 were identified as *O. ips*, 62 as *O. minus,* and 59 as *Sphaeropsis sapinea*. The remaining 183 strains were only tentatively assigned to several species of the genus *Leptographium* Lagerberg and Melin (in Lagerberg et al. 1927) (see below).

Ophiostoma ips (Rumb.) Nannf. Sv. Skogsvardsf. Tidskr. 32: 408. 1934

This economically important, bark-beetle associated sapstain fungus is regularly recorded in North America (Seifert 1993), but its actual pathogenic capability is variously reported. While South African studies demonstrated it can cause lesions, it was not considered a major threat to pines (Wingfield and Marasas 1980; Zhou et al. 2002). However, elsewhere it is reported to cause damage to various pine species either alone or in combination with other fungi (Mathre 1964; Lieutier et al. 1989*a*, 1989*b*; Nevill et al. 1995; Otrosina et al. 1997).

Both *Ceratocystis montia* (Rumb.) Hunt (1956) and *Ceratocystis adjuncti* Davidson (1978) were synonymized with *O. ips* under the name *Ceratocystis ips* (Rumb.) C.Moreau (1952) by Upadhyay (1981). However, Hausner et al. (1993*b*, 1993*c*) suggested strongly that all these species were distinct, and recently Kim et al. (2003) confirmed the separation of *O. ips* and *O. montium* (Rumb.) von Arx (1952) using both growth and molecular characters. While our PSB associated isolates did vary slightly morphologically, all were assignable to *O. ips* according to previous descriptions (Olchowecki and Reid 1974; Upadhyay 1981; Hutchison and Reid 1988*a*), therefore molecular analyses were not undertaken.

Ophiostoma minus (Hedg.) H. & P. Syd

Reported repeatedly from North America, *O. minus* can kill seedlings, saplings, and more mature trees of various species (e.g., Nelson 1934; Mathre 1964; Basham 1970; Owen et al. 1987), while in Europe its pathogenic potential and association with the PSB has been investigated extensively (Lieutier et al. 1989*a*, 1989*b*; Piou and Lieuter 1989; Solheim and Långström 1991; Långström et al. 1993), as has its comparative virulence against other blue-stain fungi isolated with it from Japanese red pine in Japan (Masuya et al. 2003). However, the relationship of *O. minus* with its host trees, insects, and other fungi is probably not simply one of a pathogen with its host (Paine et al. 1997; Klepzig et al. 2001).

These selected reports, plus many others, suggest the association of *O. minus* with the PSB in Ontario should be of concern, as it may represent either more than one species or two discrete sets of strains. A recent study supports the subdivision of *O. minus* into an European and a North American group (Gorton et al. 2004). However, unpublished rDNA RFLP data from ten North American strains suggest the North American group may not be homogeneous (Handel 1992). We identified our current *O. minus* strains solely on morphological criteria, and did not address its possible complexities.

Sphaeropsis sapinea (Fr.) Dyko & Sutton sensu lato

As diplodia tip blight, this species was reported on a range of pines in Ontario (Myren and Davis 1989; Myren 1990), and Hausner et al. (1999*b*) studied variation in culture and rDNA RFLP in isolates from pine and spruce species growing in Manitoba and Ontario. It is an important pathogen of a variety of pines and other conifers world wide (reviewed by Stanosz 1997), and it has been the subject of a number of genetic diversity studies (Stanosz et al. 1999; de Wet et al. 2000; Burgess et al. 2001*a*, 2001*b*; Zhou et al. 2001) including a recent report suggesting it represents two distinct species (de Wet et al. 2003). Although we identified our isolates solely by morphological characters, and did not distinguish between the morphotypes noted, the pathogenic ability of this fungus and its association with the PSB is of concern.

Leptographium spp.

Based on morphology, published descriptions, and supporting molecular data, we reported previously *L. wingfieldii*, a then presumed new introduction into North America, *L. procerum,* and *L. lundbergii* were associated with the PSB in Ontario (Hausner et al. 2002, 2003*b*), and the presence of *L. wingfieldii* was confirmed by Jacobs et al. (2004). However, within isolates assigned to each species, some strains varied significantly in key morphological characters: e.g., isolates of *L. procerum* that had conidiophores twice as long as previously reported, putative strains of *L. wingfieldii* with some conidiophore heads similar to the anamorph of *O. aureum* or some suggestive of *L. terebrantis*; plus some unusual presumed examples of *L. lundbergii*.

DNA sequence analysis of partial mt-*rnl* U11 intron sequences

Amplification of the mt-*rnl* U11 intron yielded amplicons of approximately 1.6 kb. We compared sequences from seven Ontario *Leptographium* strains with those from strains representing *L. wingfieldii, L. terebrantis*, *L. procerum*, and *O. aureum* (Table 2 and Fig. 1). Sequences from isolates of *O. penicillatum*, *O. piceae* (Münch) H. and P. Syd., and *O. novo-ulmi* subsp. *americana* Brasier & S.A. Kirk were included as outgroups.

This molecular criterion sorted the *Leptographium* strains into three statistically supported (≥80%, using bootstrap analysis) clades designated as the *L. procerum* and *L. lundbergii* groups, and the *Ophiostoma aureum* complex (see Fig. 1). The *O. aureum* complex includes *O. aureum*, a species whose anamorph resembles *L. wingfieldii,* and five Ontario *Leptographium* strains that clustered with strains identified elsewhere as *L. wingfieldii* or *L. terebrantis*. However, one morphologically distinct strain from Ontario (WIN(M) 1210) clustered with strains fitting the description of *L. procerum*.

PCR–RFLP analysis of the rDNA IGS region of strains of *Leptographium*

Gel electrophoresis of the amplified ribosomal IGS products showed a single dominant band per strain (Fig. 2A). The size of the IGS PCR products among members of the "*O. aureum* complex" ranged from 2.40 to 4.00 kb, whereas the size of the IGS region was uniform among the three

Table 3. Association of fungi with host tissues and (or) *Tomicus piniperda* in the sampled test sites.

	Fungi isolated			
Isolates derived from	*Ophiostoma ips*	*Ophiostoma minus*	*Sphaeropsis sapinea*	*Leptographium* spp.
Stained wood associated with both nuptial and brood galleries	4		33	88
Fruiting in *T. piniperda* nuptial galleries	70	56		50
Fruiting in *T. piniperda* brood galleries	2	6		15
Isolated directly from crushed, washed then crushed or mobile beetles walking on agar plates	4		15	26
Stained logs lacking beetleactivity (cut ends, mechanical etc.)			11	4
Total	80	62	59	183

tested strains of *L. procerum* (2.7 kb). The IGS amplicon for the two *L. lundbergii* strains, CBS 352.29 and NFRI 89-1040/1/3, were 3.3 and 2.7 kb, respectively. We could not amplify the IGS of strain WIN(M) 1210, so we used WIN(M) 1211 in the analysis; based both on morphology and sequence identity of their ITS regions, this strain is equivalent to WIN(M) 1210. The *O. penicillatum* strain served as an outgroup as it has a *Leptographium* anamorph that is distinct morphologically from those of either *L. wingfieldii*, *L. procerum*, or *L. lundbergii*. The RFLP analysis of the IGS amplicons used nine different endonucleases (examples of IGS restriction profiles for *Alu*I, *Taq*I, *Msp*I, and *Mbo*I are presented in Figs. 2B, 2C, 2D, and 2E, respectively). Polymorphisms were detected among members of the *O. aureum* complex, and also among the other species of *Leptographium* examined.

The dendrogram based on NJ analysis of the combined IGS–RFLP data set comprised 165 binary characters and yielded results (Fig. 3) congruent with the data in Fig. 1. The analyzed strains were clustered into three major groupings; the *L. procerum* group, the *L. lundbergii* group, and the *O. aureum* complex. However, the RFLP data provided additional resolution of the *O. aureum* complex by generating within it three potential clusters that received bootstrap support above the 80% level of confidence. One cluster comprised two Japanese strains of *L. wingfieldii* (MCC 130 and MCC 125) and a British Columbia strain identified as *L. terebrantis* (WIN(M) 468). A second cluster suggests that a putative *L. wingfieldii* strain (WIN(M) 1100) from Ontario is closely related to the ex-holotype strain of *L. terebrantis* (CBS 337.70). But the third, and largest cluster within this complex, includes *L. wingfieldii* ex-holotype and ex-paratype strains (CBS 645.89 and CBS 648.89, respectively), both isolated in Europe, plus other strains from Europe (CBS 345.90 and NFRI 88-369/11, United Kingdom and Sweden, respectively), Japan (MCC 349), and four from Ontario (WIN(M) 1103, 1120, 1192 and 1209). Both WIN(M)1192 and CBS645.89 (ex-holotype) yielded identical IGS–RFLP profiles for all nine endonucleases tested. Although RFLP analysis of the IGS region did yield both intra- and inter-specific differences, the dendrogram groupings are congruent with those observed in the mt-*rnl* U11 intron phylogenetic tree and with our morphological data. The IGS–RFLP data demonstrated the presence of intraspecific variation between the ex-holotype and paratype strains of *L. wingfieldii,* thus the IGS region might be useful in future studies of geographic and genetic variability among members of this pathogenic species complex.

Phylogenetic analysis of the rDNA ITS region

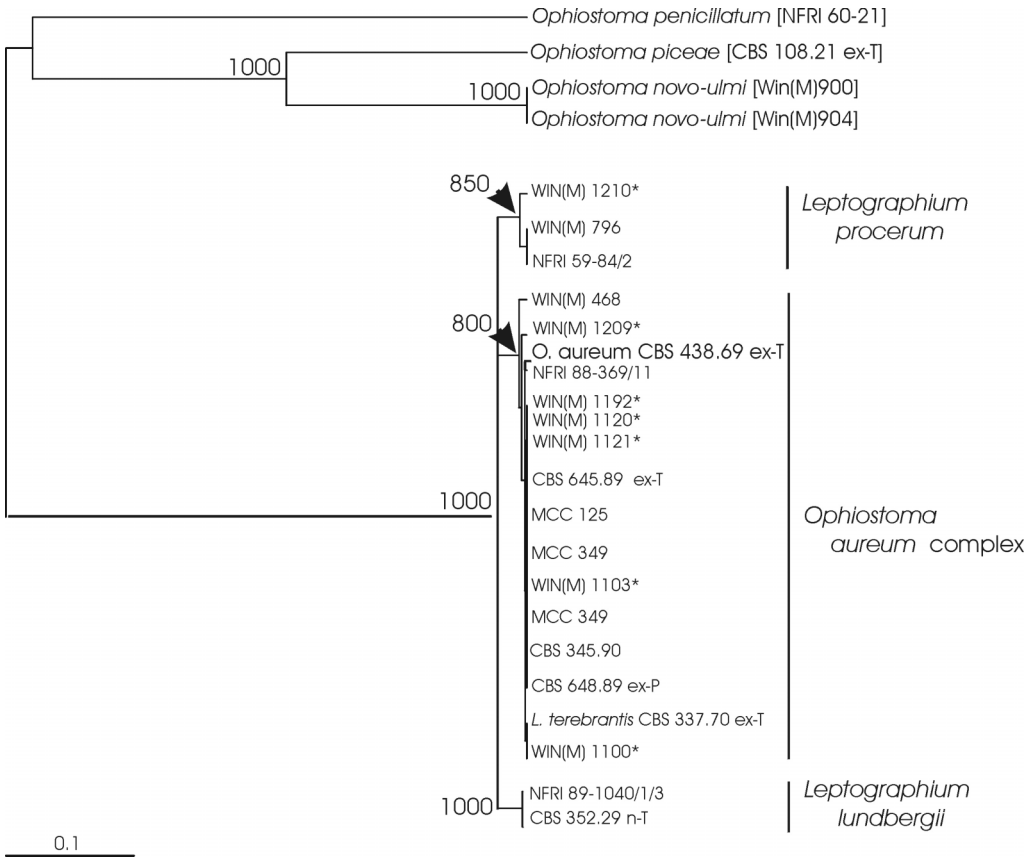
Using *O. ips* and *O. piceae* as outgroups, phylogenetic analysis of the ITS region sequences confirmed both the U11 intron sequence and IGS-RFLP analyses (Fig. 4), and supports the monophyly of strains clustered as *L. wingfieldii* at confidence levels of 98.6% (NJ analysis), 92.3% (Parsimony) and 97% quartet support (TP-ML). Sequenced strains included ex-holotype and ex-paratype strains of *L. wingfieldii*, and ex-holotype strains of both *L. terebrantis* and *O. aureum*. Analysis of this data set al.so identified at least three other monophyletic groups amongst the *Leptographium* strains tested.

Leptographium lundbergii was reported from North America (Eckhardt 2003; Eckhardt et al. 2004) and so-named Canadian strains are in CBS, DAOM, and MUCL. The ITS data set of 11 putative *L. lundbergii* strains, included an ex-holotype strain of *L. truncatum* (=*L. lundbergii* fide Strydom et al. 1997), and these strains formed a monophyletic group at 98.2% (NJ), 95% (Parsimony), and 92% quartet support (TP–ML) confidence levels, named the *L. truncatum* clade. A second set of 8 strains, including an ex-neotype strain of *L. lundbergii* (CBS 352.29), and named the *L. lundbergii* clade, was also distinct with monophyly for this clade supported at 72.2% (NJ), 94.1% (Parsimony), and 91% quartet support (TP–ML) levels. While the *L. truncatum* clade contains southern Ontario strains, WIN(M) 1246 and WIN(M) 1274, an ex-holotype strain of *L. truncatum*, and strains from Norway, Sweden, France, South Africa, and New Zealand, strains of the *L. lundbergii* clade originated only from either Sweden, Norway or Germany.

Naming strains as "*L. lundbergii*" was difficult, because the true nature of this species may have become confused as we believe the Jacobs and Wingfield (2001) description does not agree fully with the protologue of Lagerberg et al. (1927). Lagerberg et al. made no mention of the type of conidiophores described by Jacobs and Wingfield (2001), and their photographs thereof (Lagerberg et al. 1927, Fig. 5B) are quite different from those of Jacobs and Wingfield (2001, Fig. 95). Plus there are significant differences in their respective reports of spore sizes and shapes.

No authentic herbarium specimens collected, studied, or identified by either Lagerberg or Melin could be found in either Swedish herbaria or elsewhere. There are fungal strains under this name available in various culture collec-

Fig. 1. Phylogenetic estimate, based on the mt-*rnl* U11 partial intron sequence analysis, showing the potential phyletic relationships of *Leptographium* strains originating from Ontario towards other *Leptographium* species including ex-type strains of *L. wingfieldii* [CBS 645.89], *L. terebrantis* [CBS 337.70], and the neotype of *L. lundbergii* [CBS 352.29]. We are using the term *O. aureum* complex as it represents a teleomorph name, however at this point more work is required to resolve the possibility of an anamorph/teleomorph connection between *L. wingfieldii* and *O. aureum*. The tree was constructed with the NEIGHBOR program (UPGMA option; see Gibb and Hausner 2003) and evaluated using the bootstrap procedure (1000 replicates) in PHYLIP. Levels of support are indicated only for nodes that received moderate to high support (>80%). (See Table 2 for a description of the strains used.)



tions, but none appear to have been authoritatively identified by either Lagerberg or Melin. However, strains DAOM 60396 and Forintek C34, both deposited and identified by Lagerberg and received by these two laboratories in 1947, may be derived from a common ancestry.

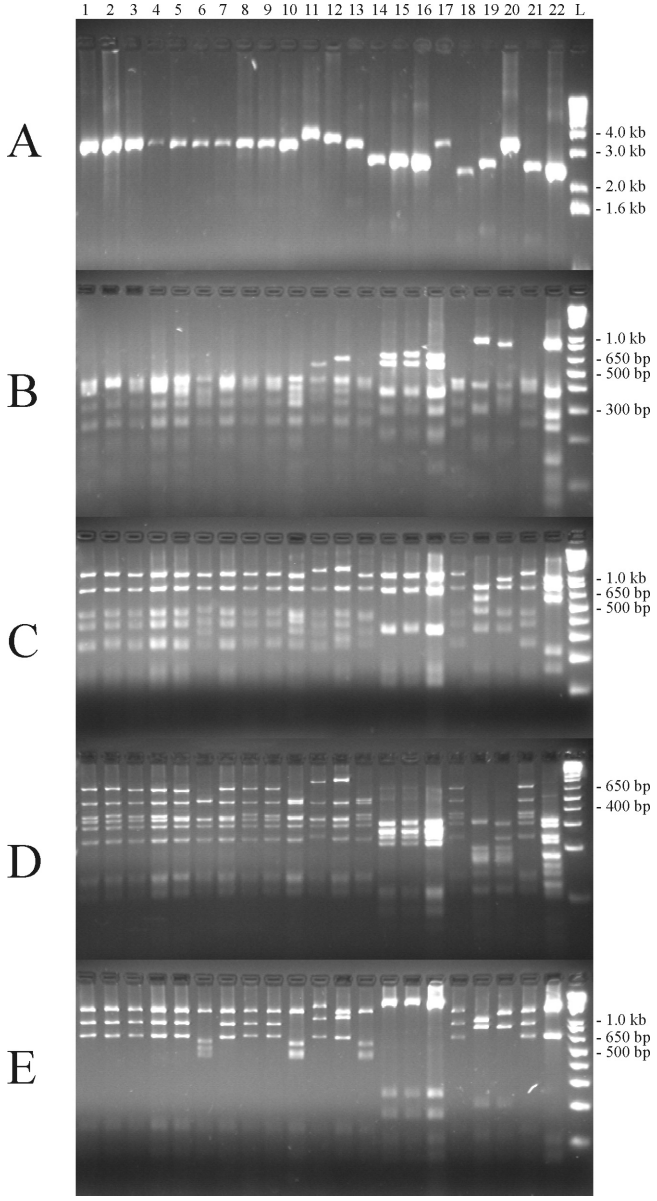
Strydom et al. (1997), following Wingfield and Gibbs (1991), reduced *L. truncatum* to synonymy with *L. lundbergii*, and neotypified *L. lundbergii* with strain CBS 352.29. To do so, they grew a culture of the latter, and dried and deposited it as the neotype in the National Fungal Herbarium, South Africa as PREM 45698. But this strain has recently been withdrawn from the CBS online catalogue.

CBS 352.29 was recorded as having been isolated, identified, and deposited by E. Melin; the date of accession given was January 1929, but no original host was cited. Jacobs and Wingfield (2001) listed the host as *Pinus* sp., with Lagerberg and Melin having made the collection in 1929. However, CBS informed us that in 1928 a letter was sent to Lagerberg requesting cultures of blue stain fungi, including *L. lundbergii*, but records indicate that he forwarded the CBS letter to Melin, who sent the cultures. However, none of the archived letters received during this exchange contain specifications on the strains he sent. Thus, it is unknown if the designated neotype represents the original concept of *lundbergii*. This matter must be addressed more fully than is appropriate herein, but for the moment we are using the name *L. lundbergii* to accommodate a number of strains we have on hand.

It was hoped that the ITS rDNA analysis of *L. lundbergii* would clarify three issues: the validity of synonymizing *L. truncatum* with *L. lundbergii*; the phylogenetic position of the *L. lundbergii* neotype with respect to other *L. lundbergii* strains from Sweden and Norway; and the species status of the Ontario PSB associated strains that morphologically resemble *L. lundbergii* sensu Jacobs and Wingfield (2001). Ten isolates tested that agreed with the description of *L. truncatum* grouped with the ex-holoypte strain of the latter into a distinct clade. However, the ex-neotype strain of *L. lundbergii* and seven other strains from Norway and Sweden formed a separate clade.

DAOM 60396 and Forintek C34 group in the clade that includes the ex-holotype of *L. truncatum* (CBS 929.85). In contrast the ex-neotype of *L. lundbergii* (CBS 352.29) is in the other clade. Could *L. truncatum* actually represent *L. lundbergii* sensu Lagerberg & Melin, and could *L. lundbergii* sensu Jacobs & Wingfield really be an undescribed species? The results also suggest *L. truncatum* is a more cosmopolitan species than *L. lundbergii* sensu Jacobs and Wingfield, with the latter being reported in Europe.

Fig. 2. PCR/RFLP analysis of PCR products that represent the nuclear ribosomal intergenic (26S/18S) region (IGS). (A) The original EF2 (IGS) PCR products. (B–E) A set of representative digests of the EF2 products with *Alu*I, *Taq*I, *Msp*I, and *Mbo*I, respectively. Note that putative strains of *L. wingfieldii* originating from Ontario have RFLP profiles that are either identical or very similar to RFLP profiles (*Cfo*I, *Mbo*I, and *Rsa*I) obtained for the extype and the paratype of *L. wingfieldii*. (See Table 2 for a description of the strains used.) The first number in brackets refers to the lane number on the agarose gel and the second number refers to strain numbers: (1) 1118; (2) 1121; (3) 1192; (4) 1130; (5) 1103; (6) 1124; (7) 1181; (8) 1182; (9)1126; (10) 1123; (11) 1100; (12) 1183; (13) 468; (14) 33; (15) 796; (16) 1210; (17) 1120; (18) 1131; (19) 1115; (20) 1209; (21) 27.



Two Ontario strains cluster within the *L. truncatum* clade, but this is not surprising. Harrington (1988, p. 36) identified two Ontario isolates from *P. resinosa* in Ontario as *L. truncatum*. Harrington still believes *L. lundbergii* and *L. truncatum* are distinct entities, for he is so quoted in the recent (01/10/05) update in Pest Reports, *Leptographium truncatum*, EXFOR Database (http://spfnic.fs.fed.us/exfor/data/pestreporter.cfm). As *L. truncatum* is a potential root pathogen of *Pinus* spp. (Harrington 1988; Wingfield et al. 1988), distinguishing it from *L. lundbergii* is essential as our results show both are vectored by the PSB in Ontario.

We accepted greater morphological variability in assigning strains to *L. wingfieldii* than did Jacobs and Wingfield (2001). They state spores are: "hyaline, aseptate, oblong with truncate bases and rounded apices, 4–6 μm × 2–3 μm, but Morelet (1988) states (translation, J.R.) "Conidia are hyaline, 1-celled, straight, cylindric-oblong to obovid, obtuse at the extremeties, sometimes truncated at the base ..... They measure 3.84–10.88 μm × 1.92–4.48 μm (mean 6.73–2.92 μm), ratio length to width 2.3:1)". Note that Morelet reports a mean length greater than Jacobs and Wingfields' maximum length, and a mean width just slightly below their maximum width. Both sets of authors record that the spores are produced in light-coloured mucilaginous droplets that become orange (Morelet 1988) or amber (Jacobs and Wingfield 2001) on aging. But depending on the strain examined, and its age, the overall spore size range could be (2.5)4–12(17) μm. The spores were produced in light coloured mucilaginous masses that became orange to dark orange on aging.

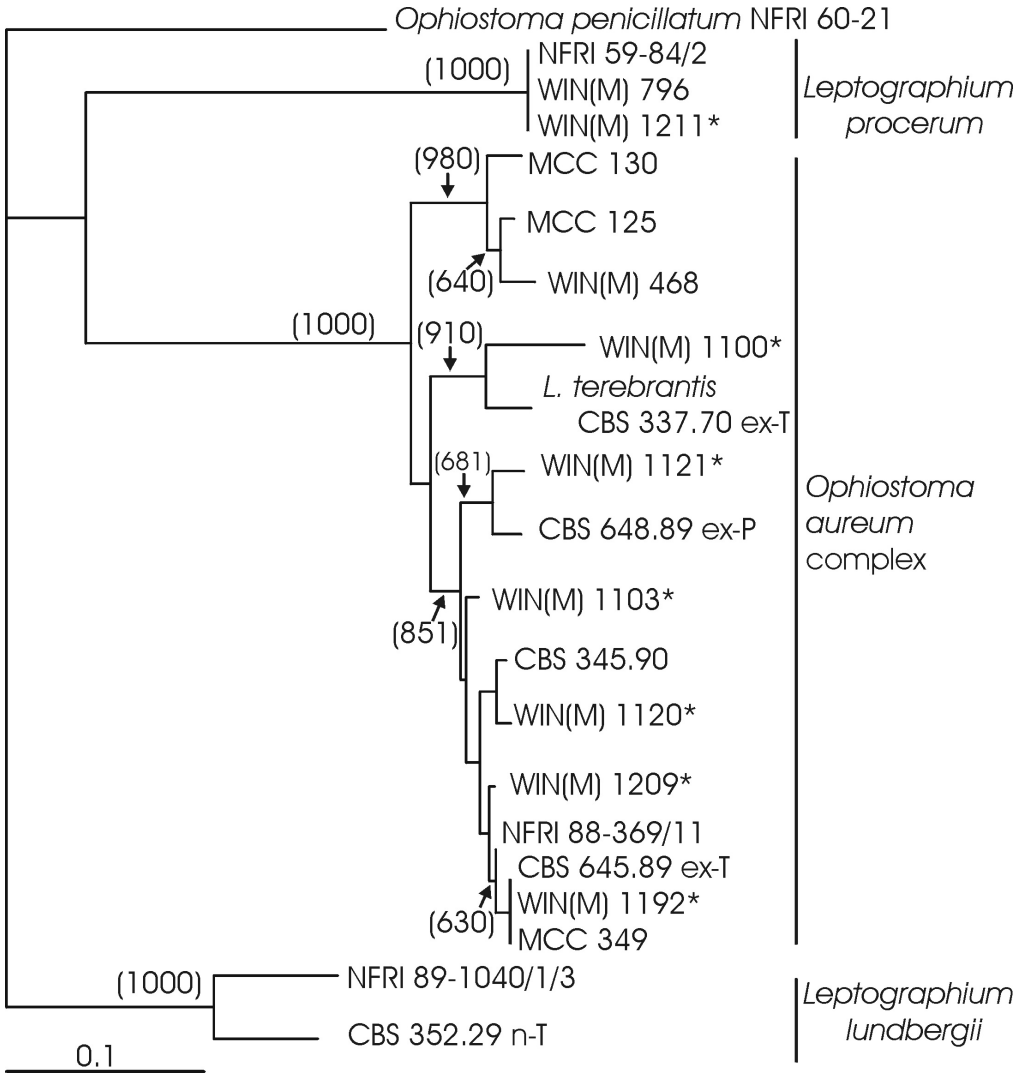
Morelet (pers. comm.) stated "it is not surprising you saw spores up to 12 μm. In fact, my given measurements in my paper was from fresh material (8-d-old culture). Whereas in older and dry material the spores are longer and wider: (5.32)6.65–12(17.3) × 2.7-5.3 μm (mean 9.15–3.7 μm)." Thus the overall spore size range we recorded for *L. wingfieldii* agrees with Morelets' own concept of this species.

Molecular analysis supported strongly the morphological assignment of some Ontario isolates *to L. wingfieldii*. The sequenced data (Figs. 1 and 4, respectively) and the IGS RFLP data (Figs. 2 and 3) show that Ontario strains WIN(M) 1209, 1192, 1121, 1103, and 1100 cannot be segregated from the ex-holotype and ex-paratype strains of *L. wingfieldii* (CBS 645.89 and 648.89, respectively). Furthermore, the molecular data suggests that *L. terebrantis*, a species capable of causing serious damage in North America (Wingfield 1986), and *L. wingfieldii* should probably be synonymized as neither DNA sequence data nor IGS–RFLP data segregated strains representing these species. Nor did morphological features expressed in our population of strains distinguish these two species. Finally the sequence data suggests a potential connection between *O. aureum*, reported from British Columbia and the Western United States, and both *L. wingfieldii* and *L. terebrantis.*

If *L. wingfieldii* and *L. terebrantis* do represent a single species, then *L. wingfieldii* is not newly introduced in North America, although the PSB might have introduced new potentially more aggressive or virulent strains, as clearly the PSB is an efficient vector of a number of fungi; the latter might allow new strains to spread faster and come into contact with other possible host species and different potential beetle vectors.

The possible genetic relationships of *L. terebrantis* with other *Leptographium* species have been studied previously (Zambino and Harrington 1992; Six et al. 2003) using a variety of isolates, but not, as far as we are aware, an ex-holotype

Fig. 3. Phylogenetic analysis of the IGS rDNA RFLP data. A total of nine different digests were used in constructing a binary (0/1) data set that consisted of 165 binary characters. The analysis clearly shows that isolates from Ontario (1100, 1103, 1120, 1121, 1192, 1209) represent *L. wingfieldii*. Bootstrap values are provided for key nodes, but RFLP data fails to fit the assumptions required for this test (see Bruns et al. 1991). Thus, the levels of branch support within the IGS–RFLP dendrogram must be viewed with caution.

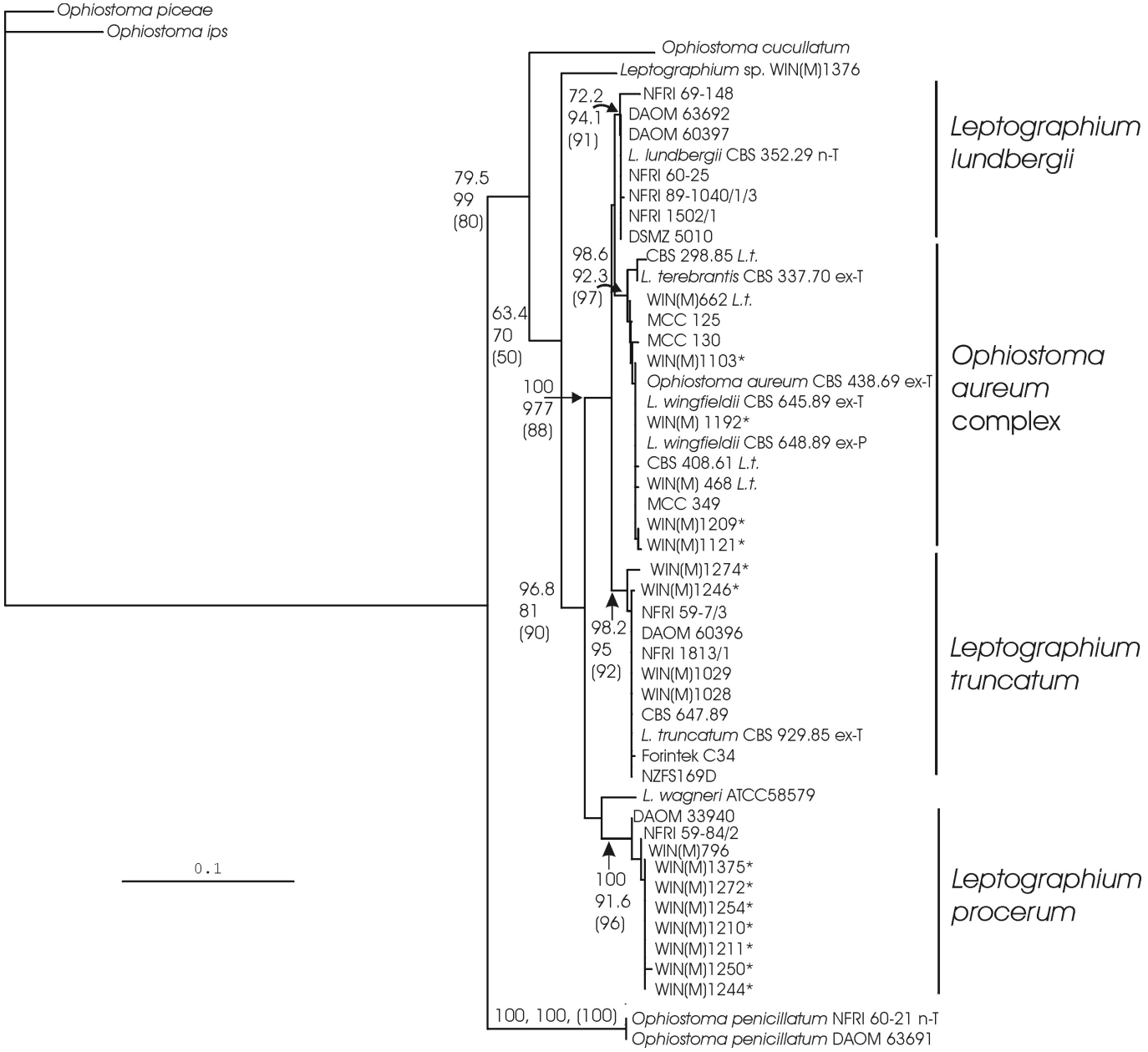


strain of *L. terebrantis*. However, they could not establish a definitive separation of *L. terebrantis* and *Ophiostoma clavigerum*, a species we did not include in our studies. Their work also shows clearly that the species concept in *L. terebrantis* warrants further investigation.

Another serious threat identified by this study is the association of *L. procerum* with *T. piniperda.* In the past, *L. procerum* has been viewed as a "southern" species, frequently associated with pine root decline diseases (Alexander et al. 1988), but one that has been infrequently reported from New York state and recorded only once from both Ontario and Quebec. Harrington has recently provided a comprehensive updated summary on this organism in Pest Reports, 01/10/05, EXFOR Database, http://spfnic.fs.fed.us/exfor/data/pestreporter.cfm.

Several of the strains recovered in Ontario produced conidiophores with stipes ranging up to 2200 μm in length, well beyond that reported by Jacobs and Wingfield (2001). Such stipes were noted in a potentially undescribed *Leptographium* species from New Zealand (Hutchison and Reid 1988*b*). Therefore, one of the New Zealand strains was included, along with strain NFRI 59-84/2, which had been verified by Harrington (1988) as authentic *L. procerum*, and one of the unusual Ontario strains (WIN(M)1210) in both the mtDNA sequence and IGS restriction analysis; later additional strains of *L. procerum* were included in the rDNA ITS phylogenetic analysis. The ITS data clusters 10 strains identified morphologically as *L. procerum* onto a single node with a level of support of 100%. This clade includes seven *L. procerum* strains recovered from either *P. sylvestris* or the PSB from Southern Ontario. Thus all such Ontario strains are *L. procerum*. It was noted that several of the Ontario strains either did not produce, or did so only faintly, the "characteristic concentric rings" formed in culture. Such growth patterns were noted by Jacobs and Wingfield (2001) in cultures of strains of *L. procerum*.

Fig. 4. Phylogenetic analysis of the ITS region sequence data. The level of confidence for the major nodes in the phylogenetic tree were determined using the bootstrap procedure in combination with first NJ and second DNAPARS analysis (SEQBOOT 1000 replicates; and CONSENSE) and the third value reported represents QP (the quartet puzzling support values; TREE puzzle). The analysis clearly shows that isolates from Ontario (1209, 1192, 1121, 1103) represent *L. wingfieldii*, and that *L. wingfieldii* could be conspecific with *L. terebrantis* and *Ophiostoma aureum*. Other sequences denoted by * represent strains of *L. truncatum* and *L. procerum* vectored by *T. piniperda* in southern Ontario.



While *T. piniperda* itself is unlikely to cause major damage to Ontario tree species, it will probably become an important vector for potentially serious fungal pathogens. This study already shows that several of its vectored fungi are known to cause serious damage in other jurisdictions. If, or when, *T. piniperda* becomes co-invasive with native bark beetles on native tree species, it is quite possible the native beetles might become important vectors of fungi they have not previously carried; indeed this already seems to have occurred (Jacobs et al. 2004). Our study also indicates that all of the PSB associated species may not be genetically homogeneous, note earlier work with *S. sapinea* and that of de Wet et al. (2003), and these may reflect more serious introductions of a variety of strains of presumed indigenous fungi, that will complicate already existing problems. Note the *O. aureum* complex as an example. If some of its members are indeed forms of a common organism and they can potentially fuse and generate new genotypes through either somatic or sexual recombination, then even more serious pathogenic strains may arise in the future. The nature of species structure in the genus *Leptographium* warrants continued serious study.

Acknowledgments

We gratefully acknowledge funding for this project as follows: operating grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to GH and PCL; NSERC strategic grant (CB); Canada Research Chair (PCL); and a Natural Resource Canada (Forestry, Sault Ste. Marie, Ont.) contribution. We are deeply grateful to Dr.O. Constantinescu for searching Swedish herbaria for possible original material of *L. lundbergii* for us; Dr. M. Morelet for the loan of the holotype specimen of *L. wingfieldii* and additional comments on that strain; Dr. C. Delatour for the loan of other specimens of *L. wingfieldii*; and Dr. J.A. Stalpers for searching the archives of CBS and reporting to us re the correspondence regarding the deposition of the neotype strain of *L. lundbergii* there. And Drs. H. Masuya and H. Solheim generously provided cultures for our use without restriction; these were most important in our study. Dr. S. Renault kindly verified French translations.

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