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Taxonomic re-evaluation of *Leptographium lundbergii* based on DNA sequence comparisons and morphology

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The genus *Leptographium* was described in 1927 and currently includes 48 species, with *L. lundbergii* as the type species. In recent years, the taxonomic status of *L. lundbergii* has not been uniformly agreed upon and it has been the topic of considerable debate. The problem was compounded by the absence of a type specimen, and the species was epitypified at a later stage. Unfortunately, the whereabouts of the epitype is now unknown. In 1983, Wingfield & Marasas described *L. truncatum*, which is morphologically similar to *L. lundbergii*. Based on DNA comparisons and similarities in their morphology, this fungus was reduced to synonymy with *L. lundbergii*. The loss of the type specimen as well as variation in the morphology of strains identified as *L. lundbergii* prompted us to re-examine the taxonomic status of this species. A number of strains from various geographic areas were studied. These include a strain of *L. lundbergii* deposited at CBS by Melin in 1929 (CBS 352.29) as well as the ex-type strain of *L. truncatum*. The strains were compared based on morphology and comparison of multiple gene sequences. Three genes or genic regions, ITS2 and part of the 28S gene, partial β-tubulin and partial elongation factor 1-α were compared. Strains currently identified as *L. lundbergii*, represented a complex of species. Strains initially described as *L. truncatum* clustered separately from other *L. lundbergii* strains, could be distinguished morphologically and should be treated as a distinct taxon. *L. lundbergii* is provided with a new and expanded description based on a neotype designated for it. A third group was also identified as separate from the main *L. lundbergii* clade and had a distinct *Hyalorhinocladiella*-type anamorph, described here as *H. pinicola* sp. nov.

INTRODUCTION

Leptographium is one of several anamorph genera associated with the genus *Ophiostoma* (Wingfield, Seifert & Webber 1993). Species in this group include the causal agents of tree diseases and most cause sap stain, particularly in conifers (Harrington & Cobb 1988, Wingfield *et al*. 1993). *Leptographium* was described by Lagerberg & Melin (1927) as a monotypic genus to accommodate the penicillately branched fungal strains isolated from the stained sapwood of pines and spruce. *L. lundbergii* was designated as the type species. Lagerberg, Lundberg & Melin (1927) commented on the similarities between *Leptographium* and *Scopularia*. They considered the description of the latter genus to be vague and thus reduced *Scopularia* to synonymy with *Leptographium*. *Scopularia* is also a later homonym of *Scopularia* Lindl., therefore, an illigitimate name (Shaw & Hubert 1952).

In their monograph, Jacobs & Wingfield (2001) treated 46 *Leptographium* species and restricted the genus to anamorphs of *Ophiostoma*. These fungi were also closely related based on sequence data for the ITS2 and 28S ribosomal gene region (Jacobs, Wingfield & Wingfield 2001). Because these fungi are morphologically similar, they have become increasingly difficult to identify and most recent studies defining species have relied strongly on DNA sequence data (Jacobs *et al*. 2004, Kim *et al*. 2004, Masuya *et al*. 2004, Zhou *et al*. 2004).

It has recently become apparent that there are discrepancies surrounding the interpretation of the type species, *L. lundbergii*. Some studies (e.g. Hausner, Reid & Klassen 2000, Hausner *et al*. 2003) have noted that there are differences in the characters described for *L. lundbergii* (Lagerberg *et al*. 1927) and those presented by Jacobs & Wingfield (2001).

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Leptographium truncatum (syn. *Verticicladiella truncatum*) was described from roots of *Pinus* spp. infested by *Hylastes* spp. in South Africa (Wingfield & Marasas 1983, Wingfield 1985). *L. truncatum* is characterised by typical *Leptographium* conidiophores that terminate in conidiogenous cells producing small, broadly truncate conidia, which at the time of the description were considered to be the main distinguishing character of this species. Wingfield & Gibbs (1991) speculated that *L. truncatum* was possibly a synonym of *L. lundbergii*, and the original cultures of *L. truncatum* were curated in the Centraalbureau voor Schimmelcultures as *L. lundbergii*, suggesting that other mycologists recognised a similarity between *L. truncatum* and *L. lundbergii*. Because they were unable to locate any original dried reference specimen linked to the name *L. lundbergii*, Wingfield & Gibbs (1991) chose not to treat the matter further. However, they did note that a culture of *L. lundbergii* collected by Melin (CBS 352.29), showed some resemblance to strains of *L. truncatum*.

L. truncatum was formally reduced to synonymy with *L. lundbergii* by Strydom, Wingfield & Wingfield (1997). These authors based their findings on a comparison of a small section of the ITS2 and 5.8S region of the ribosomal DNA for isolates of *L. truncatum* and CBS 352.29 (=CMW 217) deposited in the CBS by Melin. A dried culture of isolate CBS 352.29 was deposited in the collection of the Plant Protection Research Institute (PREM 50548) and later designates a neotype for *L. lundbergii* (Strydom *et al*. 1997).

When Jacobs & Wingfield (2001) revised *Leptographium*, *L. truncatum* and *L. lundbergii* were treated as synonyms. For the illustrations and photos, the ex-type strain of *L. truncatum* was used, because the neotype (PREM 50548) could not be located. In their study of the phylogenetic relationships within *Leptographium*, Jacobs *et al*. (2001) only used one strain of *L. lundbergii*. This strain was the same as that used in the monograph of Jacobs & Wingfield (2001), which had originally been identified as *L. truncatum*.

The current taxonomic status of *L. lundbergii* is in disarray. The strain (CBS 352.29) examined by Wingfield & Gibbs (1991) no longer sporulates, making morphological comparisons impossible. Furthermore, the neotype of *L. lundbergii* (PREM 50548) based on strain CBS 352.29 collected by Melin has been lost (I. Rong, per. comm.). The typification of *L. lundbergii* is fundamental to taxonomic studies of this genus, and we recently acquired new cultures (NFRI 60-25, NFRI 69-148), which have enabled us to re-evaluate the name and concept. One of these strains (NFRI 60-25) was collected by Aino Käärik in Sweden, who made a number of collections of this species from pine in that country (Mathiesen 1950, Mathiesen-Käärik 1953). In this study, we consider the taxonomic status of *L. lundbergii* by comparing strains assigned to this taxon from different geographic origins based on morphology and DNA comparisons.

MATERIALS AND METHODS

Source of strains

Strains used in this study were collected from various geographic areas and are maintained in the fungal genetic resource collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria (Table 1). Duplicates are also housed in the collection of the Norwegian Forest Research Institute (Skogforsk), and representative isolates of taxonomically important strains have been deposited with the CBS. The holotype specimen of *L. truncatum* (PREM 45698; culture CBS 929.85) was also included in the study.

Phylogenetic analyses

Strains (Table 1) for the phylogenetic study were grown on Potato Dextrose agar (PDA) for 10 d at 25 °C. DNA extractions from pure cultures were prepared using a modification of the method described by Möller *et al*. (1992) (Jacobs *et al*. 2004). 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 internal transcribed spacer (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 done 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, Bt2a and Bt2b (Glass & Donaldson 1995) for the β-tubulin gene and EF1F and EF2R for the elongation factor 1-α gene (Jacobs *et al*. 2004). PCR products were purified using the Qiaquick PCR purification kit (Qiagen) and sequenced using the Big Dye terminator cycle sequencing premix kit (Applied Biosystems) on an ABI PRISM 3100 automatic sequencer (Perkin Elmer Applied Biosystems). Sequence contigs were assembled using Sequence Navigator

Table 1. Source of *Leptographium* and *Hyalorhinocladiella* strains.

Species	CMW no.	Alternative no.	GenBank accession no.	Orgin	Host	Collector
L. lundbergii	217	CBS 352.29	DQ062065[a] DQ061999[b] DQ062032[c]	Europe	*Pinus* sp.	*E. Melin*
	2190	NFRI 69-148 PREM 58624	DQ062066[a] DQ062000[b] DQ062033[c]	Norway	*P. sylvestris* infested by *Pissodes pini*	*F. Roll-Hansen*
	17265	NFRI 2003-113/1 PREM 58631	DQ062064[a] DQ061998[b] DQ062031[c]	Estland	*Picea abies*	*O. Olsen & V. Timmermann*
	17266	NFRI 2003-113/3 PREM 58632	DQ062070[a] DQ062004[b] DQ062037[c]	Estland	*P. abies*	*O. Olsen & V. Timmermann*
	17264	NFRI 60-25 PREM 58623	DQ062068[a] DQ062002[b] DQ062035[c]	Sweden	*P. sylvestris*	*A. Käärik*
	17267	NFRI 2003-114/2 PREM 58633	DQ062067[a] DQ062001[b] DQ062034[c]	Estland	*P. abies*	*O. Olsen & V. Timmermann*
L. truncatum	2402		DQ062051[a] DQ061985[b] DQ062018[c]	Canada	*P. resinosa*	*J. Juzwik*
	28	PREM 45698 CBS 929.85	DQ062052[a] DQ061986[b] DQ062019[c]	South Africa	*P. taeda*	*M. J. Wingfield*
	29	PREM 45697	DQ062053[a] DQ061987[b] DQ062020[c]	South Africa	*P. taeda*	*M. J. Wingfield*
	644		DQ062058[a] DQ061992[b] DQ062025[c]	UK	*P. sylvestris* infested by *Hylastes*	*J. Gibbs*
	836		DQ062059[a] DQ061993[b] DQ062026[c]	UK	*P. sylvestris* infested by *Hylastes*	*J. Gibbs*
	21	PREM 45896	DQ062056[a] DQ061990[b] DQ062023[c]	New Zealand	*P. strobus*	*M. Dick*
	30	PREM 45699 CBS 927.85 CBS 116349	DQ062054[a] DQ061988[b] DQ062021[c]	New Zealand	*P. strobus*	*M. Dick*
	7662		DQ062055[a] DQ061989[b] DQ062022[c]	South Africa	*P. patula*	*X. Zhou*
	2850		DQ062057[a] DQ061991[b] DQ062024[c]	New Zealand	*P. strobus*	*M. Dick*
H. pinicola	2398	PREM 58621	DQ062060[a] DQ061994[b] DQ062027[c]	Canada	*P. resinosa*	*J. Juzwik*
	2399	PREM 58622	DQ062061[a] DQ061995[b] DQ062028[c]	Canada	*P. resinosa*	*J. Juzwik*
	1874		DQ062062[a] DQ061996[b] DQ062029[c]	Japan	*P. densiflora*	*M. J. Wingfield*
	1873	PREM 58620	DQ062063[a] DQ061997[b] DQ062030[c]	Japan	*P. densiflora*	*M. J. Wingfield*

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

(Applied Biosystems), aligned in ClustalX (Thompson *et al*. 1997) and manually adjusted in PAUP* v.4.0b10 (Swofford 2001).

For all data sets, phylogenetic relationships were inferred using distance analysis in PAUP* v.4.0b10 (Swofford 2001). Characters were treated as unweighted in the analysis and gaps were treated as missing data. A single tree for each dataset was obtained using neighbour-joining analysis with an uncorrected P-distance. Trees obtained from the analysis were rooted to midpoint. Confidence levels were estimated with a Bootstrap analysis (1000 replicates) using the neighbour-joining 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 partition homogeneity test (Farris *et al*. 1995) as well as a Templeton Nonparametric Wilcoxon Signed Ranked Test (Kellogg, Apples & Mason-Gamer 1996) was performed.

A Bayesian Markov Monte Carlo analysis (Larget & Simon 1999) was performed to test the confidence for the tree nodes in the distance analysis. The analysis was done using MrBayes 2.01 (Huelsenbeck & Ronquist 2001). The algorithm ran for 500 000 generations and every tenth tree was sampled. Four cold chains were run simultaneously and the trees sampled before convergence of the Markov chains were discarded in all the analysis as the burn in period.

Morphological comparisons

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 °. Fungal structures were mounted on slides in 85% lactic acid and examined using phase or differential interference contrast microscopy.

RESULTS

Phylogenetic analyses

Amplification of the ITS2 and 28S region resulted in fragments of approximately 900–1000 base pairs (bp). The aligned data set consisted of 622 characters of which 535 were constant. A single neighbour-joining tree was obtained and compared with the data of Strydom *et al*. (1997) (data not shown). DNA sequences of the ITS2 and 28S region proved to be very conserved among the strains examined in this study. Analysis of this gene region placed the *Leptographium lundbergii* strains in three groups. The first group includes the ex-type strain of *L. truncatum* (CMW 28) as well as additional strains previously identified as *L. truncatum*. These strains form a distinct group separate from the *L. lundbergii* strains. The strain collected by Melin (CBS 352.29) resided in a group together with the strain collected by Käärik in 1952 (NFRI 60-25) from a location in Sweden, approximately 100 km from the site at which the original type strain had been collected, and strains collected in the neighbour countries Estland and Norway (Table 1). A third and distinct clade aggregated strains with a distinct *Hyalorhinocladiella* anamorph. The major groups in these trees are supported by high bootstrap values and high posterior probability values for the respective nodes. Some partitioning was evident within the groups, but these branches are not well supported by bootstrap or posterior probability values.

Amplification of a part of the β-tubulin gene resulted in fragments of approximately 400–500 bp. The 3′ and 5′ ends of the sequences were trimmed in order to align them with existing data from a previous study (Jacobs *et al*. 2004; Table 2) and the aligned data set consisted of 480 characters of which 161 were constant. Four ambiguously aligned regions (241 bp) were identified and excluded from the analysis. These regions were replaced by weighted, coded characters (Lutzoni *et al*. 2000). The topology of the resulting tree is similar to that of the ITS2/28S dataset. Three distinct groups are apparent in the tree. These encompass strains of *L. truncatum*, those of *L. lundbergii* and those of the *Hyalorhinocladiella* sp. These three groups are supported by high bootstrap and posterior probability values. Considerably less variation was observed within the groups than was the case with the ITS2/28S-dataset (data not shown).

Amplification of a part of the EF 1-α gene resulted in fragments of approximately 900–1000 bp. The aligned data set consisted of 882 characters of which 324 were constant. Eight ambiguously aligned regions were identified (477 bp in total) and excluded from the analysis. These regions were replaced by weighted, coded characters (Lutzoni *et al*. 2000). The EF 1-α gene has large introns including a considerable amount of phylogenetic information and clear differences could be observed between different species. As was case with the ITS2/28S and β-tubulin data sets, the tree resulting from analysis of the EF 1-α data set placed the strains under consideration in three strongly supported taxonomic groups (data not shown).

The partition homogeneity test indicated that the β-tubulin dataset could not be combined with that of the ITS or the EF 1-α datasets, although the trees resulting from analysis of the separate sets were almost identical. Because the β-tubulin dataset is characterised by a high degree of homoplasy, we assumed that the low $P =$ values ($P = 0.002$ and $P = 0.001$, respectively) were the result of significant homoplasy in the β-tubulin dataset (Carbone, Anderson & Kohn 1999). The Templeton Nonparametric Wilcoxon Signed Ranked test, however, showed that there are no significant

Table 2. Strains of *Leptographium* and *Ophiostoma* used for molecular comparison.

Species	Strain no.	ITS	β-tubulin	Elongation 1-α
L. douglasii	CMW725	AY553380[a]	AY534928[b]	AY536174[c]
	CMW2078	AY553381[a]	AY534929[b]	AY536175[c]
L. neomexicanum	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]
	CMW60	AY553388[a]	AY534936[b]	AY536182[c]
L. aenigmaticum	CMW2199	AY553389[a]	AY534937[b]	AY536183[c]
	CMW2310	AY553390[a]	AY534938[b]	AY536184[c]
O. robustum	CMW2805	AY553396[a]	AY534944[b]	AY536190[c]
	CMW668	AY553397[a]	AY534945[b]	AY536191[c]
L. aureum	CMW709	AY553413[a]	AY534961[b]	AY536207[c]
	CMW714	DQ062071[a]	DQ062005[b]	DQ062038[c]
L. pyrinum	CMW509	AY553414[a]	AY534962[b]	AY536208[c]
	CMW169	DQ062072[a]	DQ062006[b]	DQ062039[c]
L. yunnanensis	CMW5304	AY553415[a]	AY534963[b]	AY536209[c]
	CMW5152	DQ062073[a]	DQ062007[b]	DQ062040[c]
L. wingfieldii	CMW2095	AY553400[a]	AY534948[b]	AY536194[c]
	CMW2096	AY553398[a]	AY534946[b]	AY536192[c]
	CMW2019	AY553399[a]	AY534947[b]	AY536193[c]
L. pineti	CMW3831	DQ062076[a]	DQ062010[b]	DQ062043[c]
	CMW3837	DQ062077[a]	DQ062011[b]	DQ062044[c]
L. americanum	CMW495	DQ062079[a]	DQ062013[b]	DQ062046[c]
	CMW2929	DQ062078[a]	DQ062012[b]	DQ062045[c]
L. abietinum	CMW2817	DQ062080[a]	DQ062014[b]	DQ062047[c]
	CMW3083	DQ062081[a]	DQ062015[b]	DQ062048[c]
L. laricis	CMW1980	DQ062074[a]	DQ062008[b]	DQ062041[c]
	CMW2014	DQ062075[a]	DQ062009[b]	DQ062042[c]
L. pinidensiflorae	CMW5158	DQ062082[a]	DQ062016[b]	DQ062049[c]
	CMW5162	DQ062083[a]	DQ062017[b]	DQ062050[c]

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

differences between the trees resulting from the different data sets and the datasets could be combined.

The combined dataset consisted of 1972 characters of which 1025 were constant. Twelve ambiguous regions were identified (701 bp in total) and excluded from the dataset. These regions were replaced by weighted, coded characters (Lutzoni *et al*. 2000). The tree resulting from analysis of the combined data set had an identical topology to those derived from analysis of the individual data sets (Fig. 1). The strains considered to represent *L. truncatum*, *L. lundbergii* and the undescribed *Hyalorhinocladiella*, clustered into the three taxonomic groups supported by high bootstrap and high posterior probability values for the respective nodes.

Morphology and ecology

Close examination of the strains in this study (Table 1) revealed that there are three different morphological groups amongst isolates of the *Leptographium lundbergii* complex, including *L. truncatum*. These morphological differences reflected exactly the results of the DNA comparisons. The three groups include strains previously identified as *L. truncatum* that can be distinguished from *L. lundbergii* by their taller conidiophores and the smaller almost round conidia. In our view, this is the most defining character separating these species. In the absence of adequate reference material, we propose a neotype for *L. lundbergii* based on the Swedish material deposited at the Norwegian Forest Research Institute by Käärik (NFRI 60-25). The isolate (CBS 352.29) collected by Melin has not been chosen even though it has an identical DNA sequence to isolate NFRI 60-25. This is because isolate CBS 352.29 does not sporulate and it would thus represent a poor neotype. We also provide a comprehensive description of *L. truncatum* based on the ex-type specimen (CMW 28, CBS 929.85) and reinstate this species as a distinct taxon.

TAXONOMY

Leptographium lundbergii Lagerberg & Melin, *Svensk Skogsvardsf. Tidskr*. **25**: 249 (1927). (Figs 2–13)

Synonym: *Scopularia venusta* Preuss, *Linnaea* **24**: 134 (1851); *nom. dub., nom. illegit*.

Conidiophores occurring singly arising directly from the mycelium, mostly twisted, rarely erect, macronematous, mononematous, (76–)118–279(–357) μm in length, rhizoid-like structures absent. *Stipes* light olivaceous, cylindrical, simple, 1–3-septate, (9–)12–114(–270) μm long, apical cell not swollen, 3–6 (–8) μm wide at base, basal cell occasionally swollen. *Conidiogenous apparatus*

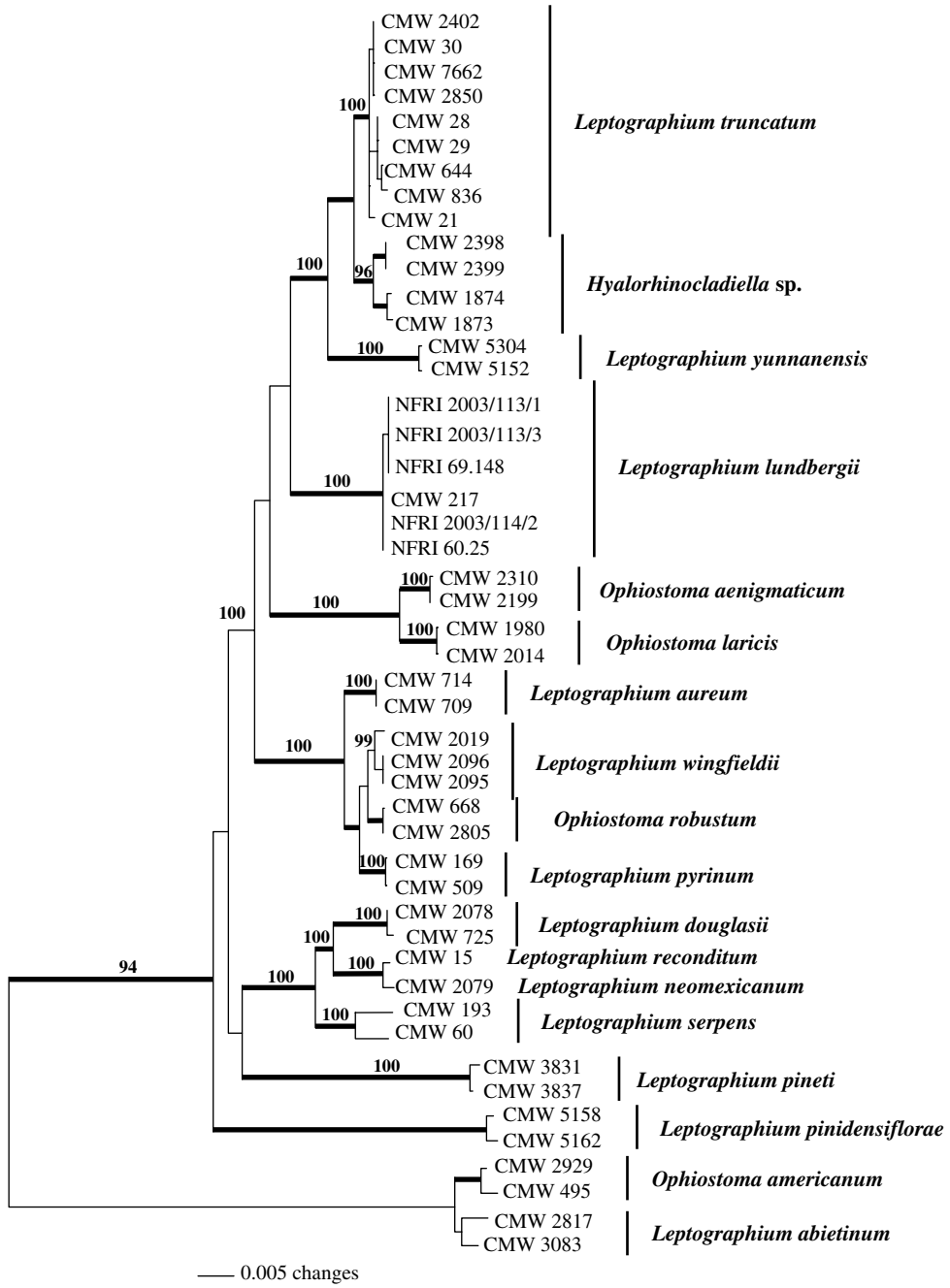


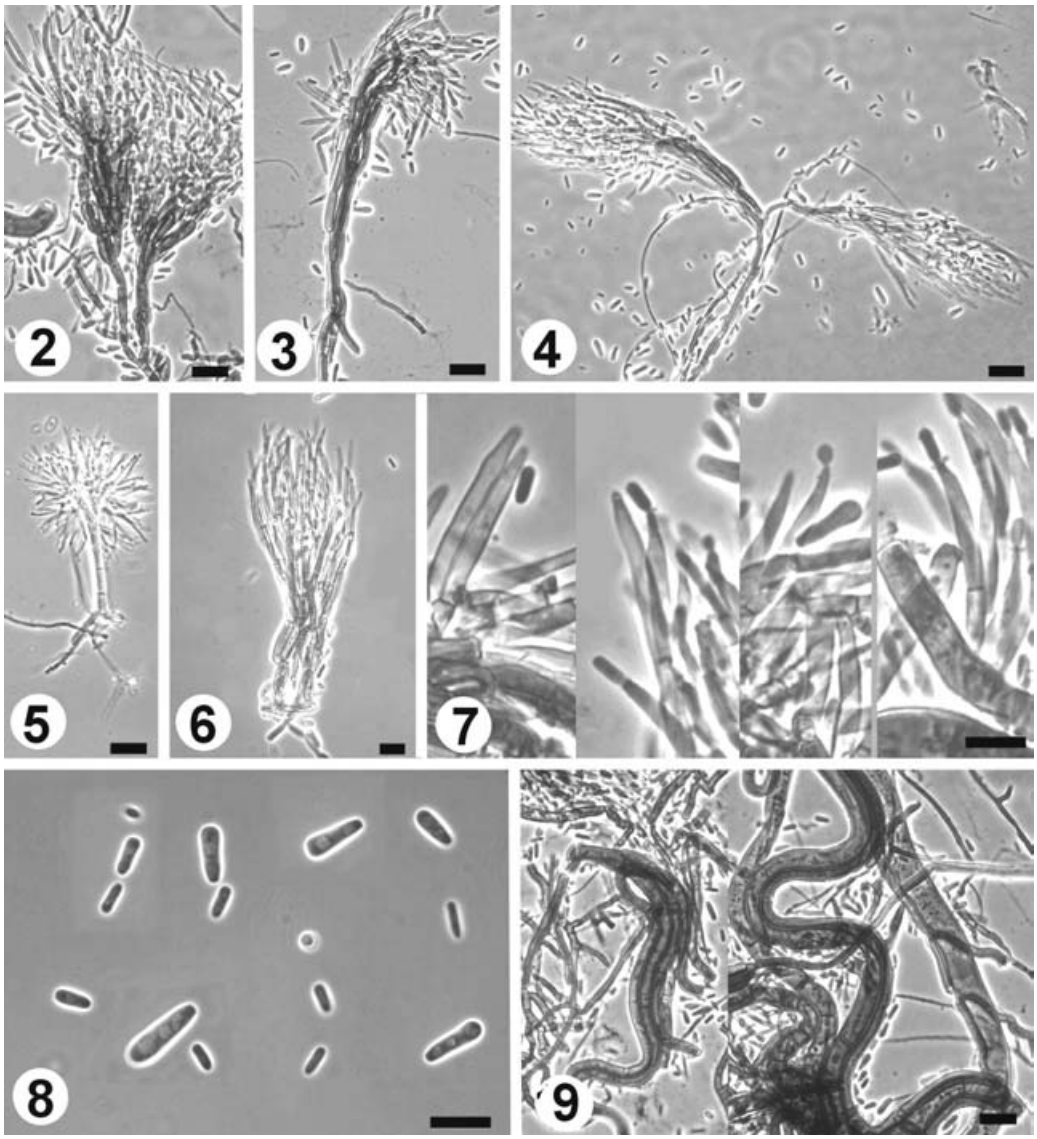
Fig. 1. Neighbour-joining tree derived from analysis of the combined dataset. Dark branches have bootstrap values above 85. Posterior probabilities for the nodes are indicated above branches.

(57–)82–215(–288) μm long, excluding the conidial mass, with multiple series of cylindrical branches. *Primary branches*, 2–3, light olivaceous, smooth, cylindrical, 0–2-septate, (13–)19–31(39) μm long and (3–)4–7(–9) μm wide, arrangement of the primary branches on the stipe-type B (more than two branches) (Jacobs & Wingfield 2001), *other branches* hyaline to light olivaceous, 0–1-septate, (11–)15–22(–26) μm long, (3–)4–7(–10) μm wide. *Conidiogenous cells* discrete, 2–3 per branch, cylindrical, tapering slightly at the apex, (17–)21–33(–41) μm long and 2–5 μm wide. Conidium development occurring through replacement wall building with holoblastic ontogeny and percurrent proliferation and delayed secession giving the false impression of sympodial proliferation (Minter, Kirk & Sutton 1982, 1983, Van Wyk, Wingfield & Marasas 1988). *Conidia* hyaline, aseptate, broadly ellipsoid with truncate bases and rounded apices, (6–)7–11(–15) × 2–4 μm.

Colonies reaching 12 mm diam. in 4 d at 25 ° on 2% MEA. *Hyphae* submerged in agar with abundant aerial mycelium, smooth, serpentine, occasionally constricted at the septa, 2–7(–19) μm wide.

Specimens examined: **Sweden**: *Uppland*: Skutskär, on *Pinus sylvestris* board, Dec. 1952, *A. Käärik* (PREM 58623, NFRI 60-25, CMW 17264) – ***neotypes hic designatus.***

Cultures examined: **Europe**: on *Pinus* sp., 1929, *E. Melin* (CMW 217, CBS352.29). – **Estonia**: Pärnu, on *Picea abies*



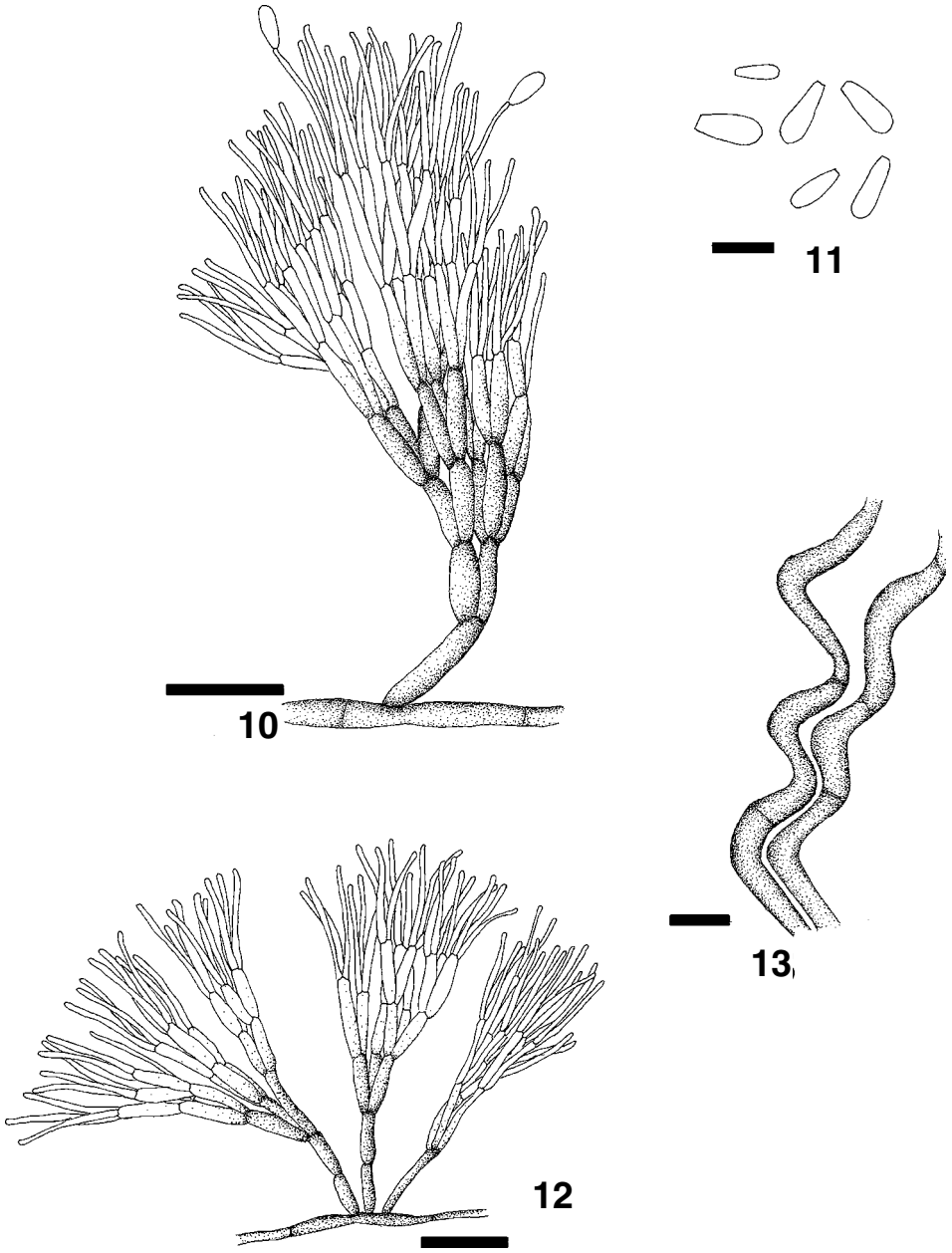
Figs 2–9. Light micrographs of the morphological characters of *Leptographium lundbergii* (NFRI 60-25). **Figs 2–6.** Variation in conidiophore morphology. Bar = 20 μm. **Fig. 7.** Conidiogenous cells showing annelidic conidium development and delayed secession of conidia. Bar = 10 μm. **Fig. 8.** Oblong conidia with broadly truncated ends. Bar = 10 μm. **Fig. 9.** Serpentine hyphae. Bar = 20 μm.

timber, 17 Nov. 2003, *O. Olsen & V. Timmermann* (NFRI 2003/113/1, CMW 17265); *loc. cit.* 17 Nov. 2003, *O. Olsen & V. Timmermann* (NFRI 2003/113/3; CMW 17266); *loc. cit.* 17 Nov. 2003, *O. Olsen & V. Timmermann* (NFRI 2003/114/2, CMW 17267). – **Norway**: on *Pinus sylvestris* board, F. Roll-Hansen, (PREM 58624, NFRI 69-148, CMW 2190).

Leptographium truncatum (M. J. Wingf. & Marasas) M. J. Wingf., *Trans. Br. mycol. Soc.* **85**: 92 (1985). (Figs 14–18)

Basionym: *Verticicladiella truncata* M. J. Wingf. & Marasas, *Trans. Br. mycol. Soc.* **80**: 232 (1983).

Conidiophores occurring singly or in groups of up to six, arising directly from the mycelium, erect, macronematous, mononematous, (90–)246–409(–685) μm in length, rhizoid-like structures absent, occasionally knob-like growths present. *Stipes* light olivaceous, cylindrical, simple, 1–16-septate, (35–)214–306(–635) μm long, 5–7 μm wide below primary branches, apical cell not swollen, 2–5(–6) μm wide at base, basal cell occasionally swollen. *Conidiogenous apparatus* (35–)42–85(–150) μm long, excluding the conidial mass, with 2 to 3 series of cylindrical branches. *Primary branches*, 2–3, light olivaceous, smooth, cylindrical, 0–2-septate, (9–)11–16(–21) μm long and (2–)3–5(–6) μm wide, arrangement of the primary branches on the stipe-type B (more than two branches) (Jacobs & Wingfield 2001), *secondary branches* hyaline to light olivaceous, 0–1-septate, (7–)8–12(–13) μm long, 1–5 μm wide, *tertiary branches* hyaline to light olivaceous, aseptate, (5–)7–10(–11) μm long, 1–3 μm wide. *Conidiogenous cells* discrete, 2–3 per branch, cylindrical, tapering slightly at the apex, (10–)11–19(–25) μm long and 1–3 μm wide. Conidium development occurring through replacement



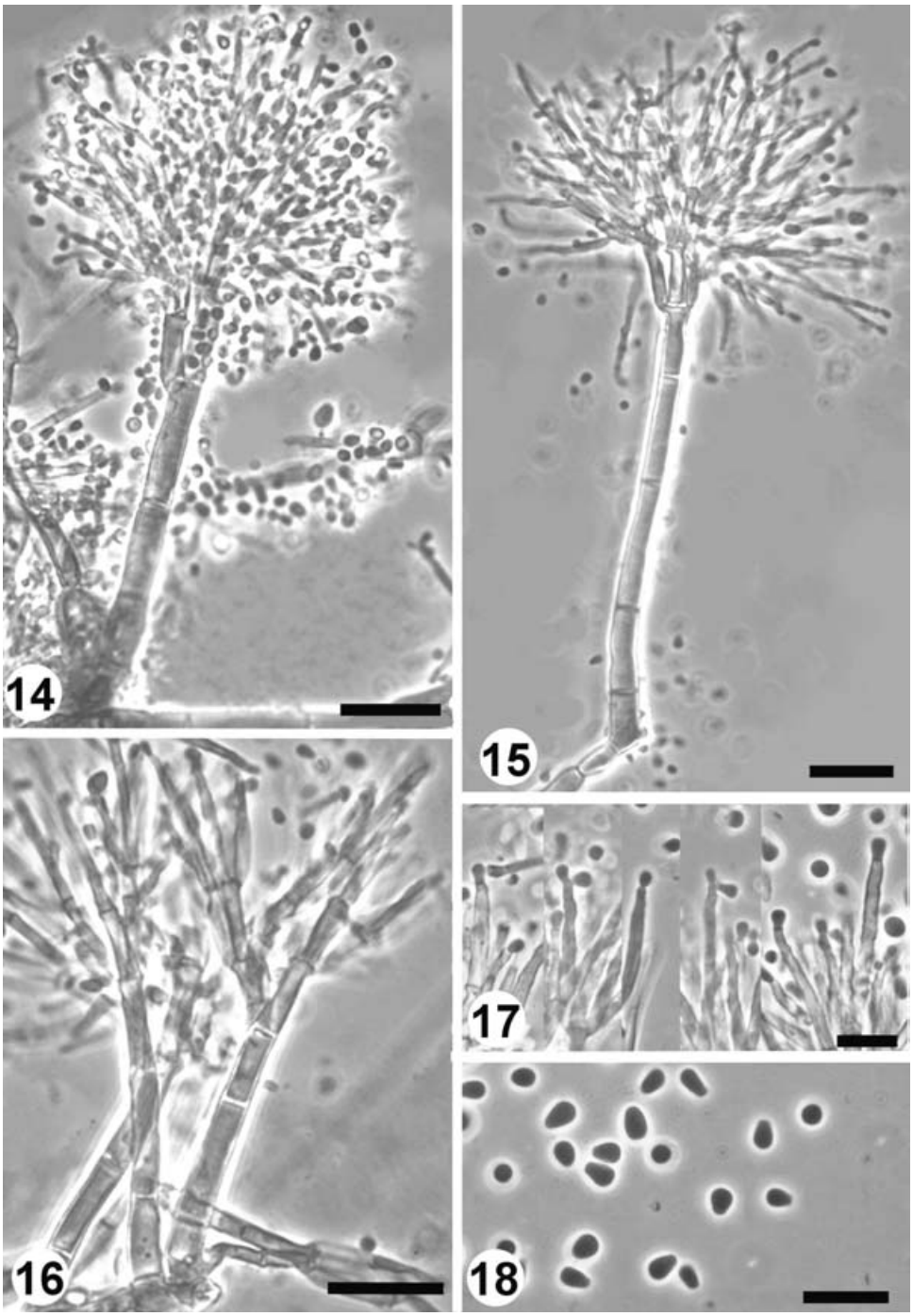
Figs 10–13. Line drawings of the morphological characters of *Leptographium lundbergii* (NFRI 60-25). **Fig. 10.** Conidiophore morphology. Bar = 20 μm. **Fig. 11.** Oblong conidia with broadly truncated ends. Bar = 10 μm. **Fig. 12.** Habit sketch of *L. lundbergii* showing the arrangement in groups. **Fig. 13.** Serpentine hyphae. Bar = 10 μm.

wall building with holoblastic ontogeny and percurrent proliferation and delayed secession giving the false impression of sympodial proliferation (Minter *et al.* 1982, 1983, Van Wyk *et al.* 1988). *Conidia* hyaline, aseptate, broadly ellipsoid with truncate bases and rounded apices, 3–5 × 2–4 μm.

Colonies reaching 24 mm in diam. in 4 d at 25 ° on 2% MEA. *Hyphae* submerged in agar with little aerial mycelium, smooth, straight, occasionally constricted at the septa, (3–)4–8(–14) μm wide.

Specimens examined: **New Zealand**: on *Pinus strobus*, 1986, *M. Dick* (PREM 45696). – Gwavas State Forest, on *Pinus strobus* roots, May 1979, *M. Dick* (PREM 45699, CBS 927.85); Horner State Forest, on *Pinus radiata* roots, May 1979, M. Dick (PREM 45700, CBS 928.85). – **South Africa**: *Sabie*: Wilgeboom seed orchard, on *Pinus taeda* roots, Dec. 1978, *M. J. Wingfield* (PREM 45698 – holotype). *East Transvaal*: Maraiti Plantation, on *Pinus taeda* roots, Feb. 1979, *M. J. Wingfield* (PREM 45697).

Cultures examined: **Canada**: Larose Forest, Bourget, on *Pinus resinosa*, *J. Juzwik* (CMW 2402). – **New Zealand**: Gwavas State Forest, on *Pinus strobus* roots, May 1979, *M. Dick* (CBS 927.85, CMW 30; on *Pinus strobus*, 1986, *M. Dick* (CMW 21; on *Pinus strobus*, 1986, *M. Dick* (CMW 2850). – **South Africa**: *Sabie*: Wilgeboom seed orchard, on *Pinus taeda* roots, Dec. 1978, *M. J. Wingfield* (CMW 28, CBS 929.85). *East Transvaal*: Maraiti Plantation, on *Pinus taeda* roots, Feb. 1979, *M. J. Wingfield* (CMW 29). On *Pinus patula*, Dec. 2000, *X. Zhou* (CMW 7662). – **UK**: *Suffolk*: Thetford, on *Hylastes* sp. from *Pinus strobus*, 1986, *J. Gibbs* (CMW644, CMW 836).



Figs 14–18. Light micrographs of the morphological characters of *Leptographium truncatum* (CMW 28). **Figs 14–16.** Variation in conidiophore morphology. Bar = 20 μm. **Fig. 17.** Conidiogenous cells showing annelidic conidium development and delayed secession of conidia. Bar = 10 μm. **Fig. 18.** Pyriform conidia with broadly truncated ends. Bar = 10 μm.

Hyalorhinocladiella pinicola K. Jacobs & M. J. Wingf., **sp. nov.** (Figs 19–28)

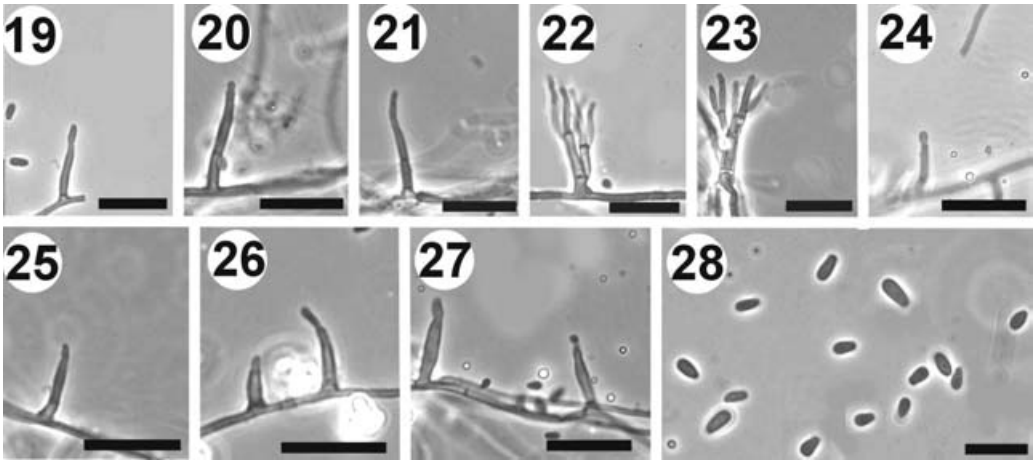
Etym.: Pine dwelling.

Coloniae 5 mm diametro in 4 diebus ad 25 ° in 2% MEA attingentes. Hyphae in agaro immersae, cum mycelio aerio abundante, laeves, rectae, interdum ad septas constrictae, 2–7 μm latae. Conidiophorae haud vel minime ramosae, (11–)15–32(–48) μm longae, conidiogenese annelidico. Conidia illis L. truncati valde similia, parva, obtusa vel obovoidea, extremitatibus late truncatis, 3–5(–6) × 2–3 μm.

Typus: **Canada**: on *Pinus resinosa*, *J. Juzwik* (PREM 58621 – holotypus).

Colonies reaching 5 mm in diam. in 4 d at 25 ° on 2% MEA. *Hyphae* submerged in agar with abundant aerial mycelium, smooth, straight, occasionally constricted at the septa, 2–7 μm wide.

Four strains initially identified as *L. truncatum*/*L. lundbergii* by M. J. Wingfield grouped close to *L. truncatum* although in a distinct group. This was noticeable in all three datasets. Closer morphological examination



Figs 19–28. Light micrographs of the morphological characters of the *Hyalorhinocladiella pinicola* (CMW 2398). **Figs 19–27.** Variation in conidiophore morphology. Bar = 20 μm. **Fig. 28.** Pyriform conidia with broadly truncated ends. Bar = 10 μm.

of these strains revealed that the conidiophores resemble those of the genus *Hyalorhinocladiella*, even though the conidia resemble those of *L. truncatum*. The conidiophores occur as unbranched or minimally branched structures (11–)15–32(–48) μm and produce conidia through percurrent proliferation of the conidiogenous cells. The conidia resemble those of *L. truncatum* closely and are small, obtuse to obovoid with broadly truncate ends, 3–5(–6) × 2–3 μm.

Specimens examined: **Canada**: on *Pinus resinosa*, *J. Juzwik* (PREM 58621, CMW 2398); on *Pinus resinosa*, *J. Juzwik* (PREM 58622, CMW 2399). – **Japan**: *Kofu*: on *Pinus densiflora* infested with *Hylastes* sp., *M. J. Wingfield* (PREM 58620, CMW 1873); *loc. cit.* on *Pinus densiflora* infested with *Hylastes* sp., *M. J. Wingfield* (CMW 1874).

DISCUSSION

Leptographium lundbergii is the type species of a genus of ecologically important fungi, including plant pathogens, insect associates and agents of blue stain in timber. Confusion regarding the circumscription of this species must necessarily impacts negatively on the understanding of the genus as a whole. At the time of description of *L. lundbergii* it was not necessary to designate type specimens for newly described species. Lagerberg *et al.* (1927) based their description on a single specimen from Sweden, which has to be interpreted as the holotype but is lost. Sadly they did not lodge a culture in a recognised collection. In the present study, we have had the privilege of studying an isolate from Sweden, collected by Käärik who was clearly familiar with *L. lundbergii*. We believe that our DNA sequence comparison, as well as morphological observations should secure the name for *L. lundbergii*. Furthermore, cultures and specimens have been deposited in a number of collections, which will hopefully avoid confusion in the future. In this study we have shown that strains of *L. lundbergii* from Sweden and neighbouring countries (NFRI 60-25, NFRI 69-148, NFRI 2003-113/1, NFRI 2003-113/3, NFRI 2003-114/2) form a well-resolved phylogenetic group.

Many of the more recent references to *L. lundbergii* in the literature refer to *L. truncatum* as now clarified. Thus, isolates from South Africa (Wingfield & Knox-Davies 1980, Zhou *et al.* 2001a, 2002), the UK (Gibbs & Inman 1991, Wingfield & Gibbs 1991), New Zealand (Shaw & Dick 1980), and the USA (Eckhardt, Jones & Klepzig 2004) reflect collections of *L. truncatum*. This fungus appears to have a relatively wide distribution globally, where it is typically associated with root inhabiting insects such as *Hylastes* spp. (Wingfield & Knox-Davies 1980, Shaw & Dick 1980, Wingfield & Marasas 1983, Kaneko & Harrington 1990, Gibbs & Inman 1991, Wingfield & Gibbs 1991, Zhou *et al.* 2001a, 2002, Eckhardt, Jones & Klepzig 2004).

Results of this study show that the decision to reduce *L. truncatum* to synonymy with *L. lundbergii* (Strydom *et al.* 1997) was incorrect. The decision to recommend this synonymy firstly arose from the original isolates of *L. truncatum* being accessioned by the Centraalbureau voor Schimmelcultures as representing *L. lundbergii*. Subsequently, DNA sequence comparisons showed that isolates of *L. truncatum* were the same as those of a single isolate of *L lundbergii* collected by Melin (CBS 352.29), and one of the original authors of the paper describing the species (Lagerberg *et al.* 1927). The use of DNA sequence comparisons at the time of the study of Strydom *et al.* (1997) was rare for fungal species. In retrospect is clear that the data were biased by the use of a small amount of DNA sequence (less than 300 bp) and the lack of sufficient *Leptographium* isolates to give a clear result. In this study, we have made use of additional gene sequences for different species. Our results provide clear evidence that *L. lundbergii* and *L. truncatum* represent discrete species. These results are furthermore supported by a study comparing isozyme profiles of different species in *Leptographium* (Zambino & Harrington 1992). Although they only used a single isolate of *L. lundbergii* from Sweden (NFRI 69-148),

Table 3. Comparison of *Leptographium lundbergii* and related species.

Characters	*L. lundbergii* (sensu Lundberg & Melin 1927)	*L. lundbergii* (NFRI 60-25)	*L. lundbergii* (sensu Jacobs & Wingfield 2001)	*L. truncatum*	*L. truncatum* (CMW 28)	*L. pini-densiflorae*	*Hyalorinocladiella* spp.	*L. yunnanensis* sp.
Anamorph type	*Leptographium*	*Leptographium*	*Leptographium*	*Leptographium*	*Leptographium*	*Leptographium*	*Hyalorhinocladiella*	*Leptographium*
Conidiophore length	Not reported	(76–)118–279(–357) µm	(90–)246–409(–685) µm	45–550 µm (combined stipe and condiogenous apparatus)	(58–)96–150(–197) µm	54–170 µm	(11–)15–32(–48) µm	74–233 µm
Primary branch number		2–4	2–3	2–4	2–3	2–4	0–2	2–3
Primary branch arrangement type		Type B	Type B	Type B	Type B	TypeB	NA	Type B
Rhizoids	Absent	Absent	Absent	Present	Absent	Absent	Absent	Absent
Conidium shape	Oblong with truncated ends	Oblong with truncated ends	Broadly ellipsoid with truncated ends	Pyriform to subglobose	Pyriform to subglobose	Oblong to ellipsoid	Pyriform to obovoid	Obovoid with truncated ends
Conidium size	8–12 × 4–6 µm	(6–)7–11(–15) × 2–4 µm	3–5 × 2–4 µm	3–11 × 2–4.5 µm	3–6 × 2–4 µm	2.5–13 × 1–3 µm	3–6 × 2–3 µm	4–11 × 2–6 µm
Hyphae	Convoluted	Convoluted	Straight	Straight	Straight	straight	Straight	Granular, straight
Hosts	Pine	*Pinus sylvestris*	*Pinus strobus* *Pinus radiata* *Pinus taeda*	*Pinus taeda* *Pinus strobus*	*Pinus taeda*	*Pinus densiflora*	*Pinus densiflora*	*Pinus yunnanensis* *Pinus densata* *Pinus kesiya*
Associated insects	None		*Hylastes* sp.	*Hylastes angustatus*		*Tomicus piniperda*	*Hylastes* gallery	*Tomicus piniperda*
References	Lagerberg *et al.* (1927)	This paper	Jacobs & Wingfield (2001)	Wingfield & Marasas (1983)	This paper	Masuya *et al.* (2000)	This paper	Zhou *et al.* (2001)

strains from *L. truncatum* clearly grouped separately from the *L. lundbergii* strain.

In contrast to *L. truncatum*, *L. lundbergii s. str.* is known only from Europe (Mathiesen 1950, Mathiesen-Käärik 1953, von Pechmann, Graessle & Wutz 1964, Kotynkova-Sychrova 1966, Dowding 1970, 1973, Käärik 1975, Hallaksela 1977) where it appears to be an agent of sapstain on pine and spruce. No insect associates have been defined for this fungus, which also appears to sporulate poorly in culture. This is ironic as it is morphologically very different to the majority of *Leptographium* species, that have well developed and in many cases elegant, erect mononematous conidiophores, terminating in impressive series of apical branches (Jacobs & Wingfield 2001). Nonetheless, this study has confirmed that *L. lundbergii* is phylogenetically related to other species of *Leptographium*, including *Ophiostoma* spp. with *Leptographium* anamorphs.

We included a suite of isolates that have been included in collections as *L. lundbergii* (Wingfield & Marasas 1983, Strydom *et al.* 1997). A third group was observed that is different to the *L. truncatum* and the *L. lundbergii* groups. Our comparisions show that this fungus represents a distinct taxon, which morphologically, is a species of *Hyalorhinocladiella*. We have thus provided the name *H. pinicola* for it. *Hyalorhinocladiella* is a common anamorph genus in *Ophiostoma* and the absence of pronounced denticals on the conidiophore distinguish this genus from the similar *Sporothrix*. Thus far, only the type species (*H. minuta-bicolor*) has been provided with a species name (Upadhyay & Kendrick 1975). As the species in this genus is clearly different to the type species and could also not be linked to any teleomorph in *Ophiostoma*, we opted to name it as a distinct taxon.

L. lundbergii as defined in this study is morphologically different from *L. truncatum*. Colonies of strains of *L. lundbergii* tend to grow more slowly than those of *L. truncatum*. Furthermore, sporulation in the former fungus is sparse and the conidiophores occur in 'untidy clumps' at the surface of the mycelium in cultures. These conidiophores are most similar to those of *L. yunnanensis* described from *Pinus* spp. in China. Interestingly, *L. yunnanensis* is also phylogenetically closely related to *L. lundbergii* and *L. truncatum* (Zhou *et al.* 2001b). The conidiophores of *L. lundbergii* tend to be stunted with short stipes and large conidiogenous apparatuses. These compare well with the photograph of this species in the original description in Lagerberg *et al.* (1927), and a more recent treatment of the species by Hallaksela (1977) based on an isolate from *Picea abies* in Finland. The conidia of *L. lundbergii* are about twice the size of those of *L. truncatum* and in both these species, the conidia tend to be broadly truncate.

Masuya *et al.* (2000) suggested that their newly described *L. pini-densiflorae* collected from *Pinus* spp. in Japan is morphologically similar to *L. lundbergii*. However, they compared their new species with strains

of *L. lundbergii* that are recognised as *L. truncatum* in the present study. Nonetheless, their species is different to both *L. truncatum* and *L. lundbergii* (Table 3, Fig. 1). The major difference between these species is the rounded ends of the conidial bases in *L. pini-densiflorae*, compared with the truncated bases found in conidia of *L. lundbergii* and *L. truncatum* (Lagerberg *et al.* 1927, Wingfield & Marasas 1983, Masuya *et al.* 2000).

Zhou *et al.* (2001a) conducted a study to identify the ophiostomatoid fungi associated with three different non-native pine-infesting bark beetles in South Africa. *L. lundbergii* was one of the species that was found to occur in association with bark beetles on pines in South Africa. The description of Jacobs & Wingfield (2001) was used for the identification of this species and it was, therefore, based on strains that we now consider to be *L. truncatum*. They also found that their strains were only weakly pathogenic (Zhou *et al.* 2001a), which was also reported by Kaneko and Harrington (1990) for *L. truncatum* in Japan. *Leptographium lundbergii* and *L. truncatum* are clearly ecologically very different fungi. *Leptographium lundbergii*, as it is defined in this study, has only been found in Europe where it has been associated with stained sapwood on pine and spruce (Bakshi 1950, Mathiesen 1950, Mathiesen-Käärik 1953, Kotyncova-Sychrova 1966, Dowding 1970). In contrast, *L. truncatum* appears to have a more cosmopolitan distribution and has mostly been reported associated with root disease on pines in South Africa, New Zealand and Japan. In the UK this species is associated with bark beetle galleries in wind-blown pines (Gibbs & Inman 1991, Wingfield & Gibbs 1991).

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