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Ribosomal DNA Sequence Comparison of *Leptographium lundbergii* and *L. truncatum* and neotypification of *L. lundbergii*

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Summary

Recent studies have shown that *Leptographium lundbergii* and *L. truncatum* are morphologically similar. This has led to suggestions that the species, which both occur in Europe and are associated with European scolytid bark beetles, might represent a single taxon. Synonymising these two species based on their similar morphology has been resisted in the past. This is primarily due to the fact that *L. lundbergii* is the type species of the genus and it was felt that such a decision should be based on more intensive study. PCR amplified DNA fragments of isolates of *L. lundbergii* and *L. truncatum* from numerous geographic origins were compared on the basis of the DNA sequence from the ITS2 region and part of the 5.8S gene of the rRNA operon. Phylogenetic analyses show that *L. lundbergii* and *L. truncatum* isolates group together and separate from the outgroups, *L. wingfieldii, Ceratocystis fimbriata* and *C. albofundus*. Based on these data and the morphological similarity of the two species, we conclude that *L. truncatum* is a synonym of *L. lundbergii*.

Key word: rRNA, ITS2, *Leptographium lundbergii*.

Introduction

Species belonging to the conidial genus *Leptographium* Lagerberg & Melin are dematiaceous and have mononematous conidiophores that terminate in a conidiogenous apparatus made up of a series of branches. Conidiogenous cells arise from the terminal branches and give rise to hyaline conidia that accumulate in slimy masses (Lagerberg et al., 1927). Most species of *Leptographium* are associated with insects that infest trees and they usually sporulate in the galleries of these insects, where gloeoid spore masses facilitate dispersal (Harrington and Cobb, 1988). Furthermore, numerous species of *Leoptographium* are associated with root diseases of conifers (Harrington and Cobb, 1988; Wingfield et al., 1988).

Leptographium was established for *L. lundbergii* isolated from discoloured pine lumber in Sweden (Lagerberg et al., 1927). The complex history of the genus has been reviewed by Harrington (1988). Hughes (1953) established *Verticicladiella* Hughes as distinct from *Leptographium* based on sympodial conidium development in the former, and annellidic conidium development in the latter genus. Following the same trend, Kendrik (1961) established *Phialocephala* for fungi resembling *Leptographium* and *Verticicladiella* but in which conidia developed from phialides. Kendrick (1962) also monographed *Verticicladiella* excluding species of *Leptographium* and *Phialocephala*. Later, Wingfield (1985) reduced *Verticicladiella* to synonymy with *Leptographium* based on electron microscopic evidence showing only one pattern of conidium development in the two genera studied.

Members of the *Leptographium* complex are common anamorphs of some species of *Ophiostoma* Sydow & Sydow (De Hoog and Scheffer, 1984; Harrington, 1988; Wingfield, 1993). Species of *Ophiostoma*, including those with *Leptographium* states, share the distinguishing characteristic of having rhamnose and cellulose in their cell walls (Weijman and De Hoog, 1975; De Hoog and Scheffer, 1984). They also tolerate high concentrations of cycloheximide in culture media (Harrington, 1981). *Leptographium* isolates without known teleomorph connections also share the cell wall and cycloheximide tolerance characters (Harrinton, 1988; Wingfield, 1993).

WINGFIELD and MARASAS (1983) described *Verticicladielle truncata* Wingfield & Marasas as a new species associated with pine-infesting bark beetles in South Africa and New Zealand. This fungus was compared with all other species of *Verticicladiella* and found to be distinct, particularly on the basis of its truncate conidia. However it was not compared with *Leptographium* spp. at that time, or later when it was transferred to *Leptographium* (WINGFIELD, 1985). In a study of *Leptographium* spp. associated with pine-infesting bark beetles in England, WINGFIELD and GIBBS (1991) noted that *L. truncatum* was morphologically indistinguishable from a culture identified as *L. lundbergii* and deposited in the culture collection of the Centraalbureau voor Schimmelcultures (CBS) at Baarn, Netherlands, by Melin, one of the original authors of the species.

The temptation to synonymise *L. lundbergii* and *L. truncatum* was resisted by WINGFIELD and GIBBS (1991). This was firstly because *L. lundbergii* is the type species of the genus *Leptographium*, and it was felt that a thorough comparison with a number of isolates would be wise. Moreover, no type material existes for *L. lundbergii* thus necessitating neotypification, and this should require thorough study of this species. The aim of this study was, therefore, to compare a number of isolates of *L. lundbergii* and *L. truncatum* from various surces that conform to the description of LAGERBERG et al. (1927). Comparisons are based on the sequence data from the internal transcribed spacer 2 (ITS2) region and part of the 5.8S rRNA gene. This region was chosen particularly because of the high degree of sequence variation within the ITS region among species (CHAMBERS et al., 1986). Also, sequence data from this region have clarified many fungal taxonomic problems (OTSUKA et al., 1983; BAURA et al., 1992; VILJOEN et al., 1993).

Material and methods

Three isolates of *L. lundbergii*, six isolates of *L. truncatum*, one isolate of *L. wingfieldii* Morelet and one isolate of *Ceratocystis fimbriata* Ell. & Halst. and *C. albofundus* Wingfield, de Beer & Morris as outgroups were included in this study (Table 1). The isolates of *L. lundbergii* and *L. truncatum* chosen for comparison were all morphologically indistinguishable and had diverse origins (Table 1). Isolates of *L. truncatum*, included a culture of the type of this species (ATCC 58100) from South Africa. Two of the three isolates (LUND 1 and LUND 2) of *L. lundbergii* originated in Sweden and were collected by Melin and Lagerberg, respectively. They were the senior and junior authors of the original description of *L. lundbergii* and it was, therefore, assumed that these are authentic cultures of the species. *L. wingfieldii* was included to as an outgroup (SWOFFORD and OLSEN, 1990) because, like *L. lundbergii*, it is associated with insects that infest pine, and is superficially similar to the latter species in morphology. *Ceratocystis fimbriata* and *C. albofundus* were included as more distant outgroups.

Table 1. List of the cultures, their origins and hosts.

Isolate nr.	Culture Collection nr.	Species	Origin	Host	Collector	Reference
TRUN1	CMW21, PREM45896	*L. truncatum*	New Zealand	*Pinus radiata*	M. Dick	WINGFIELD (1985)
TRUN2	CMW28, ATCC58100	*L.truncatum*	South Africa	*P. taeda*	M. J. Wingfield	WINGFIELD (1985)
TRUN3	CMW2402, C168	*L. truncatum*	Canada	*P. resinosa*	J. Juzwik	ZAMBINO and HARRINTON (1992)
TRUN4	CMW2398, C288	*L. truncatum*	Canada	*P. resinosa*	J. Juzwik	ZAMBINO and HARRINTON (1992)
TRUN5	CMW2408, C400	*L. truncatum*	Japan	*P. densiflora*	S. Kaneko	KANEKO and HARRINGTON (1990)
TRUN6	CMW2400, C401	*L. truncatum*	Japan	*P. thunbergii*	S. Kaneko	KANEKO and HARRINGTON (1990)
LUND1	CMW217, CBS352.29, PREM50548	*L. lundbergii*	Sweden	*P. sylvestris*	E. Melin	LAGERBERG et al. (1927)
LUND2	CMW761, *C34	*L. lundbergii*	Sweden	*P. sylvestris*	J. Lagerberg	LAGERBERG et al. (1927)
LUND3	CMW2192, ATCC22735, C59	*L. lundbergii*	Sweden	*P. sylvestris*	E. Jorgensen	
WING	CMW2096, M207	*L. wingfieldii*	France	*P. sylvestris*	M. Morelet	MORELET (1988)
FIMBR	PREM51642	*C. fimbriata*	France	*Platanus hybrida*	C. Grosclaude	GROSCLAUDE and OLIVIER (1991)
ALBOF	PREM51641	*C. albofundus*	South Africa	*Acacia mearnsii*	S. McLennan	WINGFIELD et al. (1996)

Culture collections are as follows: ATCC – American Type Culture Collection; CBS – Centraal Bureau voor Schimmelcultures; *C – Culture Collection of Forintek Canada Corporation; supplied by Dr K. Seifert, Centre for Land & Biological Resource Research, Ontario, Canada; CMW – Culture Collection of M. J. Wingfield; C – Culture Collection of T. C. Harrington; M – Culture Collection of M. Morelet; PREM – Official designation of the National Collection of Fungi, Pretoria, South Africa.

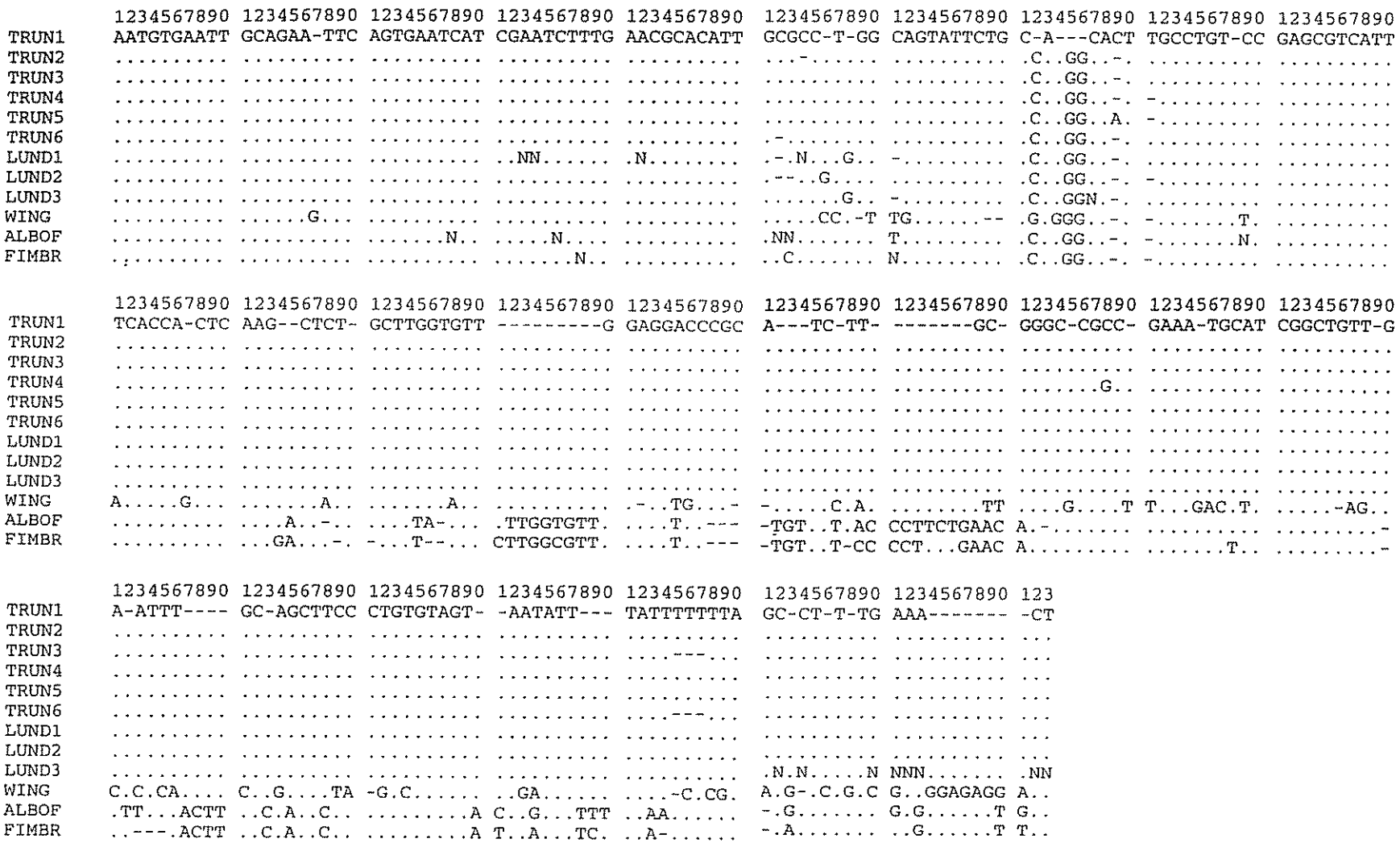

Fig. 1. Aligned sequences of a 273 base pair section of the ITS2 region and part of the 5.8S rRNA gene of six *Leptographium truncatum* isolates, three *L. lundbergii* isolates, one isolate of *L. wingfieldii, Ceratocystis fimbriata* and *C. albofundus*. Bases identical to the first *L. truncatum* sequence are indicated by a point, a dash indicates a gap in the sequence inserted in order to achieve the alignment.

Cultures were grown on malt-extract agar plates (20 g/l) overlaid with sterile cellophane sheets. The cellophane sheets, overgrown with mycelium, were then lyophilized. Mycelium was scraped from the surface of the cellophane sheets and DNA isolations were performed using the method described by VILJOEN et al. (1993).

The first internal transcribed spacer (ITS1), the 5.8S rRNA gene and the second internal transcribed spacer region (ITS2) were amplified using the primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') (WHITE et al., 1990) and CS1 (5'TAGCTGATCCGAGGTCAA3'). Some difficulty was experienced while amplifying the regions within the rRNA operon of the *Leptographium* spp. using conventional primers. The problem was overcome by synthesising a consensus sequence primer, CS1, that binds to the 5' end of the large subunit rRNA gene and represents a consensus sequence found in all ophiostomatoid fungi sequenced in our laboratory (authors, unpublished). PCR amplifications were done on a Hybaid Omnigene Temperature Cycler (Hybaid, Middlesex, UK). An initial denaturation was done at 96 °C for 5 minutes, followed by 35 cycles of 90 °C for 1 minute, 55 °C for 15 seconds and 72 °C for 30 seconds. The reaction was completed with a 72 °C chain elongation step for 5 minutes. The PCR products were visualised on a 1.7% agarose gel containing ethidium bromide, using UV light. A single DNA fragment of 550 base pairs (bp) was amplified, which includes the ITS 1 and ITS 2 regions, as well as the 5.8S rRNA gene.

The PCR products were purified using the Magic PCR Preps (Promega Corporation, Madison, USA) and sequenced using the Fmol DNA Sequencing System (Promega Corporation, Madison, USA). A 273 bp region of the amplified fragment was sequenced. Ambiguous bases were resolved sequencing both stands of DNA with the two primers CS1 and ITS 3 (5'TCGATGAAGAACGCAGC3') (WHITE et al., 1990). The sequence data were manually aligned. Phylogenetic relationships were determined by making use of the Phylogenetic Analysis Using Parsimony (PAUP) program (SWOFFORD, 1993). DNABOOT analysis (Bootstap confidence intervals on DNA parsimony) (FELSENSTEIN, 1985) was performed to assess the confidence intervals of the branch points.

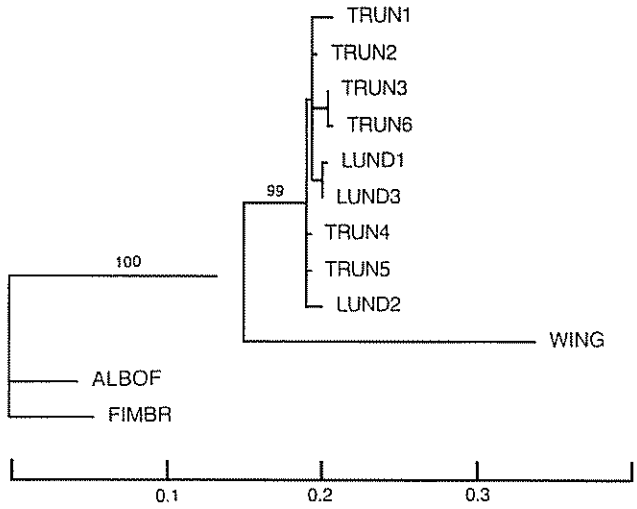

Fig. 2. The phylogram produced from the PAUP analysis based on the nucleotide sequence of the ITS2 region of isolates from *Leoptographium lundbergii* (LUND 1–3), *L. truncatum* (TRUN 1–6) and one isolate of *L. wingfieldii* (WING), *Ceratocystis fimbriata* (FIMBR) and *C. albofundus* (ALBOF). Confidence intervals of the branch points are indicated, using 100 bootstrap repeats.

Results

A single tree was produced using a PAUP analysis. Figure 2 shows the results of a Heuristic search, with *C. fimbriata* and *C. albofundus* defined as the outgroups. All the *L. lundbergii* and *L. truncatum* isolates group together, distinct from *L. wingfieldii* and the more distant *C. fimbriata* and *C. albofundus* outgroup. Using a bootstrap analysis, the branch point separating the *L. lundbergii/L. truncatum* clade from *L. wingfieldii* had a confidence interval of 99%. The confidence interval of the branch point separating the *Leptographium* clade from *C. fimbriata* and *C. albofundus* was found to be 100%. A table of the pairwise distance between the taxa are presented in Figure 3.

Below diagonal: Absolute distances
Above diagonal: Mean distances (adjusted for missing data)

		1	2	3	4	5	6	7	8	9	10	11	12
1	TRUN1	–	0.018	0.026	0.022	0.018	0.029	0.026	0.029	0.023	0.260	0.246	0.244
2	TRUN2	5	–	0.015	0.011	0.011	0.018	0.011	0.018	0.011	0.253	0.235	0.232
3	TRUN3	7	4	–	0.018	0.018	0.004	0.022	0.026	0.019	0.249	0.243	0.240
4	TRUN4	6	3	5	–	0.007	0.022	0.019	0.015	0.015	0.249	0.231	0.229
5	TRUN5	5	3	5	2	–	0.022	0.019	0.015	0.015	0.249	0.231	0.229
6	TRUN6	8	5	1	6	6	–	0.019	0.022	0.023	0.253	0.243	0.244
7	LUND1	7	3	6	5	5	5	–	0.019	0.004	0.260	0.239	0.240
8	LUND2	8	5	7	4	4	6	5	–	0.023	0.253	0.231	0.232
9	LUND3	6	3	5	4	4	6	1	6	–	0.250	0.236	0.237
10	WING	71	69	68	68	68	69	70	69	66	–	0.388	0.402
11	ALBOF	66	63	65	62	62	65	63	62	61	104	–	0.098
12	FIMBR	66	63	65	62	62	66	64	63	62	109	26	–

Fig. 3. A table of the pairwise distance between *Leptographium lundbergii* (LUND 1–3), *L. truncatum* (TRUN 1–6), *L. wingfieldii* (WING), *Ceratocystis fimbriata* (FIMBR) and *C. albofundus* (ALBOF).

Conclusion

Results of this study clearly show that isolates of *L. lundbergii* and *L. truncatum* group together as a clade distinct from *L. wingfieldii*. Furthermore, no distinct grouping occurred amongst isolates of *L. lundbergii* and *L. truncatum* and there was no pattern indicating separation of the two groups. Indeed, results indicate a greater degree of variation between the two isolates designated as *L. lundbergii*, than between certain *L. lundbergii* and *L. truncatum* isolates. This is perhaps not surprising given the fact that we were not able to distinguish among isolates based on their morphology.

There has been some concern expressed over the weighting of gaps resulting from the alignment of sequence data (ANDERSON and STASOVSKI, 1992). We, therefore, repeated the PAUP analysis where all gaps in the sequence were removed, with the remaining portion of the sequence still aligned. The analysis resulted in a similar phylogenetic tree where all *L. lundbergii* and *L. truncatum* isolates group together, with *L. wingfieldii* considerably distant from them.

ZAMBINO and HARRINGTON (1992) characterised species of *Leptographium* using isozyme analysis. These authors included an isolate purported to be of *L. lundbergii* from *Pinus sylvestris* in Norway. They did, however, note that this fungus was morphologically different from *L. truncatum* isolates, one of which had also been received as *L. lundbergii*. In the study of ZAMBINO and HARRINGTON (1992) the purported isolate of *L. lundbergii* groups relatively closely with isolates characterised as *L. truncatum* although it was also shown to be more similar to *Ophiostoma huntii* (Robinson-Jeffrey) de Hoog & Scheffer (anam. *L. huntii* Wingfield) which is unquestionably a distinct species. In this study, we have chosen not to include the Norwegian isolate examined by ZAMBINO and HARRINGTON (1992) which we believe to be more typical of *O. huntii*.

Based on the results of this study, we confirm the previous contention of WINGFIELD and GIBBS (1991) that *L. truncatum* is a synonym of *L. lundbergii*. All indications are that the isolates of *L. lundbergii* used in this study and collected by Lagerberg or Melin are representative of the fungus that these authors originally described. We, therefore, propose to neotypify *L. lundbergii* based on isolate CBS 352.29 collected by Melin. Dried cultures of this fungus have been deposited in the herbarium of the National Collection of Fungi, Private Bag X134, Pretoria 0001 (PREM 50548) to represent this neotype. Illustrations of the neotype have recently been published by WINGFIELD and GIBBS (1991) and should assist in future identifications. We also prospose the following synonymy:

LEPTOGRAPHIUM LUNDBERGII Lagerberg Melin, Svens. Skogs. Tids. 25:248 (1927)
= *Leptographium truncatum* (Wingfield & Marasas) Wingfield, Trans. Br. Mycol. Soc. 85:92 (1985)
= *Verticicladiella truncata* Wingfield & Marasas, Trans. Br. Mycol. Soc. 80:232 (1983).

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