Part 2

Chapter 1

Jacobs, K., Wingfield, M.J. and Wingfield, B.D. (1999). Phylogenetic relationships in *Leptographium* based on morphological and molecular characters. Submitted to Canadian Journal of Botany.

Phylogenetic relationships in *Leptographium* based on morphological and molecular characters

Species in *Leptographium* are characterized by mononematous conidiophores with dark stipes and conidiogenous apparatuses with complex series of branches. These fungi generally inhabit woody substrates, are associated with bark beetles (Coleoptera: Scolytidae) and are known to cause blue-stain on conifers. Few phylogenetic studies have been conducted on *Leptographium* spp. and those that have been undertaken, have been focused on a small number of species. The aim of this study was to investigate the phylogenetic relationships between species in *Leptographium* based on partial DNA operon sequences and to ascertain whether morphological characters are congruent with DNA based phylogeny. Morphological characters were, analyzed and compared with results from DNA sequence analysis. Results indicate that there are three groups within *Leptographium* based on DNA sequence analysis. There was, however, no congruence between these groups and those emerging from morphological characters. Data from this study strongly support the connection between *Leptographium* and *Ophiostoma.* They have also provided us with a non-subjective means to confirm the identity of many *Leptographium* spp. that are difficult to distinguish based on morphological characters.

Keywords: *Leptographium,* phylogeny, morphology, *Ophiostoma,* rRNA

INTRODUCTION

Species of *Leptographium* Lagerberg & Melin can be recognized by their dark mononematous conidiophores and complex conidiogenous apparatuses. Conidia are produced through annelidic conidium development and are single-celled, hyaline spores (Kendrick, 1962). Some *Leptographium* spp. are known as anamorphs of *Ophiostoma* Sydow & Sydow spp. (Harrington, 1988; Wingfield, 1993). These fungi are able to tolerate high levels of cycloheximide (Harrington, 1981) and have rhamnose and cellulose in their cell walls (Marais, 1996; Horner, Alexander & Julian, 1986).

Ophiostoma and *Leptographium* spp. mostly occur on conifers in association with insects, and particularly bark beetles (Harrington, 1988; Solheim, 1986; 1993). Most *Leptographium* spp. are known to cause blue-stain in the sapwood of lumber. Only one species is well recognized as a pathogen. This includes the three varieties of *L. wageneri* (Kendrick) Wingfield that cause black stain root disease in the western USA (Wagener & Mielke, 1961; Kendrick, 1962; Cobb, 1988). *Leptographium procerum* (Kendrick) Wingfield has been associated with a root decline disease, primarily in Eastern North America (Harrington & Cobb, 1983; Alexander, Horner & Lewis, 1988) and *L. serpens* with a root disease in pines in South Africa and Italy, but the role of these fungi in disease remains unclear (Lorenzini & Gambogi, 1976; Wingfield & Knox-Davies, 1980; Wingfield, Capretti & Mackenzie, 1988).

Several new species have been described in *Leptographium* in recent years (Wingfield, Crous & Tzean, 1994; Van der Westhuizen *et al.*, 1995; Jacobs, Wingfield & Bergdahl, 1997; Jacobs *et al.*, 1998, 1999; Webber, Jacobs & Wingfield, 1999). Some of these are unusual in that they are associated with niches such as soil and non-coniferous hosts. Several have also been described from tropical areas, which is an unusual niche for *Leptographium* spp. (Wingfield *et al.*, 1994; Webber *et*

al.,1999). This has posed some questions regarding the relatedness of species described in *Leptographium* and the phylogenetic placement of the atypical species.

Zambino and Harrington (1992) used isozymes to determine phylogenetic relationships within some *Leptographium* spp. They concluded that isozyme variation can be useful in determining relationships within the genus. In general, their data also supported morphological species groupings. In a recent study of a selected group of *Leptographium* spp., Coetsee (1999) showed an apparent correlation between conidium length and phylogenetic groupings. Although the correlation was not equivocal, it did suggest that conidium length might be used to infer phylogeny in *Leptographium*.

Sequences of the ribosomal DNA genes have proved useful in determining phylogenetic relationships within groups of Ascomycetes (Gaudet *et al.,* 1989; Okada, Takematsu & Takamura, 1997; Ward & Adams, 1998; Myburg, Wingfield & Wingfield, 1999; Witthuhn *et al.,* 1999). This is especially true for morphologically similar taxa (Glenn *et al.,* 1996; Wingfield *et al.,* 1996; Dupont, Laloui & Roquebert, 1998; O'Donnell, Cigelnik & Nirenberg, 1998, Chen, Shrearer & Crane, 1999; Myburg *et al.,* 1999; Witthuhn *et al.* 1999). In the ophiostomatoid fungi, comparison of ribosomal gene sequences has been valuable in resolving various taxonomic questions, at least at generic and ordinal levels (Hausner, Reid & Klassen, 1993a,b; Wingfield, Viljoen & Wingfield, 1999). The majority of these studies have made use of the ITS and 5.8 S ribosomal RNA operon sequences. However, the large subunit of the ribosomal gene (28S) is sufficiently conserved to allow determination of relationships between genera, but is also sufficiently sensitive to distinguish relationships between species, in many cases (Gaudet *et al.,* 1989; Yamada & Kawasaki, 1989; Yamada *et al.,* 1989; Guého, Kurtzman & Peterson, 1989; Kurtzman & Robnett, 1991; Peterson & Kurtzman, 1991; Hausner, Reid & Klassen, 1993b; Vilgalys & Sun, 1994; Wingfield *et al.,* 1994).

Various authors have attempted to correlate morphological characters and DNA sequence phylogeny, with various levels of success. Berbee and Taylor (1992) concluded that morphological characters can be misleading and are not a reflection of true relationships in ascomycetes. This was also the case with Hausner *et al.* (1992) and Wingfield *et al.* (1994) who found that relationships based on ascospore shape were not congruent with phylogenies based on DNA sequence data. They thus cautioned against the use of certain morphological characters in taxonomy. In other studies, a strong correlation has been found between relationships based on morphology and phylogeny (Kurtzman, 1993; Strydom, Wingfield & Wingfield, 1997; Jacobs and Rehner, 1998; Witthuhn *et al.,* 1998; Myburg *et al.,* 1999).

The combination and comparison of morphological with molecular characters is difficult. Hibbett and Vilgalys (1993) coded the morphological data for *Lentinus* spp. in a manner similar to that of sequence data and analyzed these data as they would sequence data. From the results they determined whether relationships based on morphology could be correlated with those determined through phylogeny. A similar approach was followed by Viljoen (1996) who studied the phylogeny of *Ceratocystis,* s.l. based on morphological characters. Patterson, Williams and Humphries (1993) concluded that comparison of phylogenies based on morphology and molecular data can only be made if large data sets of morphological characters for the organisms in question, exist.

The aim of this study was to determine the phylogenetic relatedness of species within *Leptographium* through comparison of the partial sequence of the ITS2 and 28S ribosomal RNA operon. A selected set of isolates were also used to determine the placement of *Leptographium* in a larger group of Ascomycetes. Secondly, morphological characters from a large data set were coded and analyzed. Derived trees were compared to those generated from the molecular data to determine whether relationships based on morphological characters are congruent with those generated based on DNA sequence data.

MATERIAL AND METHODS

Molecular comparisons

Representative isolates were selected for known *Leptographium* spp. (Table 1). These isolates represent the majority of the described *Leptographium* spp. Species not included in the study are *L. aenigmaticum* Jacobs, Wingfield & Yamaoka, *L. neomexicanus* Wingfield, Harrington, & Crous and *L. serpens* (Goidanich) Arx. These species were omitted because we failed to amplify the DNA of the desired region despite repeated attempts. Where possible, and within the limitations of our budget, more than one isolate per species was included.

DNA extractions were performed using a modification of the technique described by Raeder & Broda (1985). Genomic DNA was extracted from two-week-old cultures grown in liquid ME (malt extract). This was done by grinding a small amount of mycelium in liquid nitrogen to a fine powder and adding 1.0 µl Extraction buffer (1 % CTAB). This was then incubated in a 60 °C waterbath for 1 hour. Proteins were removed with phenol and chloroform (1:1), followed by a series of chloroform steps, until the interface was clean. The DNA was precipitated with cold 100 % ethanol and left for 2 hours at -20 °C. This solution was then centrifuged at 13000 rpm for 30 min., the resulting pellet was washed with cold 70 % ethanol and dissolved in 100 µl sterile water. The presence of DNA was confirmed by agarose gel electrophoresis and visualized through ethidium bromide staining under the UV light.

The ITS2 (internal transcribed spacer region) and part of the large subunit (28S) of the ribosomal DNA gene were amplified using the Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1988) on a Hybaid™ Touchdown thermo cycling system (Life Sciences International, UK). The primers ITS3 (5'-GCATAGATGAAGAAGCAGC-3') (White *et al.*, 1990) and LR3 (5'-CCGTGTTTCAAGACGGG-3') (White *et al.*, 1990) were used

to amplify the required DNA fragment. Reaction volumes were 100 µl and contained 10 µl 10X PCR buffer (Boehringer Mannheim, Germany), 5 mM $MgCl_2$ (Boehringer Mannheim, Germany), 10 mM dNTP's, 20 pmol of each primer, 0.5 µl DNA and 1.75 U Expand *Taq* polymerase (Boehringer Mannheim, Germany). The PCR reaction conditions were as follows: 2 min. at 94 °C, annealing at 48 °C for 1 min., 10 s at 62 °C, 2 min. at 72 °C with an increase of 5 °C/s. This was repeated for 40 cycles and a final elongation reaction was done at 72 °C for 8 min. The resulting products were purified with the High Pure PCR product Purification Kit (Boehringer Mannheim Kit) and used in DNA sequence reactions.

Sequencing was done using the primers ITS3, LR3, LR1R (5'-AGGAAAAGAAACCAACC-3') (White *et al.*, 1990) and 404X (5'-CCCTTTCAACAATTTCAC-3') (designed based on consensus sequences of selected *Leptographium* and *Saccharomyces cereviseae* LSU sequences). Sequencing was performed on a ABI 377 automated sequencer using the Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Perkin Elmer Applied Biosystems). Sequence data were edited using Sequence Navigator (Perkin Elmer Applied Biosystems) and aligned using the alignment algorithm, CLUSTAL. Analyses were performed in PAUP* (version 4.0) (Phylogenetic Analysis Using Parsimony *and other methods) (Swofford, 1999). Gaps were treated as missing data. Analyses were performed using parsimony and heuristic search option (TBR-tribisection reconnection). Bootstrap values were determined by 100 Bootstrap replicates. Selected *Leptographium* rRNA operon DNA sequences were also compared with other genera of the Ascomycetes. Sequences for the taxa of for comparisons were obtained from genbank (Table 1).

A partition homogeneity test was performed on the different data sets (Huelsenbeck, Bull & Cunningham, 1996). This test gives an indication whether data sets can be combined, or analysed separately. Partition homogeneity tests were performed to determine whether ITS2 sequence data and LSU sequence data can be combined.

This test was also used to determine whether the morphological data set could be combined with that representing molecular data.

Morphological comparison

The data used for comparison were obtained from a morphological study of all described species in *Leptographium* (Jacobs, unpublished). Representative isolates of each species were grown on 2 % Malt extract agar (MEA) (Table1). These were compared with the original description of each species as well as with available herbarium specimens. Fungal structures for microscopic examination were mounted on glass slides in lactophenol. Fifty measurements of each relevant morphological structure were made and ranges and averages computed.

Nineteen different characters were used in the comparison. Approximately half of all described *Leptographium* spp. have been connected to a teleomorph (Harrington, 1988; Wingfield, 1993) and for this reason only anamorph characters were used. Morphological characters included conidiophore length, stipe length and conidiogenous apparatus length, morphology of the hyphae and stipe, the presence or absence of rhizoids, primary branch pattern and number of primary branches. They also included primary, secondary, tertiary and quaternary branch length, length of the conidiogenous cells, conidium shape and length, ratio of conidium length to width, optimal growth temperature as well as host and insect associations (Table 2). Species of *Leptographium* can be divided into three groups according to the length of the conidiophore. However, the ranges of the groups overlap considerably. Therefore, the character was reduced to a multi-state character and species were coded according to their range. Similarly, there are three forms of primary branch arrangement in *Leptographium,* which we refer to as type A (only two branches), type B (two or more branches) and type C (two or more branches with a single branch that are twice to three times as broad as the others). All species of *Leptographium* can be defined in terms of one of these types. The last character

considered was the length to width ratio of conidia. All species in *Leptographium* can broadly be defined in terms of small, medium or large conidia. As in the case of the conidiophores, this character was reduced to multiple characters to incorporate the overlapping ranges for the different species.

Morphological characters were coded according to the binary coding system proposed by Viljoen (1996). Characters were defined as multi-state and coded as present (1) or absent (0) (Table 2). A matrix was compiled for the data set and data analyzed using parsimony analysis, as well as distance analysis (UPGMA) using the PAUP* v.4 program (Swofford, 1999). Weighting of taxonomically important characters was done as proposed by Viljoen (1996). These characters include conidiophore length, number of primary branches and the type of arrangement. The weight assigned to a specific character was calculated as the largest number of character states in a character divided by the number of character states in the given character, times a 100. Parsimony analysis, as well as distance analysis (UPGMA) was used for the data set. A bootstrap analysis (100 replicates) was done to calculate the bootstrap values. No outgroup taxa were used and the trees were rooted to midpoint.

RESULTS

Molecular comparison

A single band of 1500 bp was obtained for all the isolates using the primers ITS3 and LR3. Of these 645 bp (base pairs) were used for comparison. This region included the last ten base pairs of the 5.8S gene, the whole ITS2 region and the first part of the 28S gene. Results from the partition homogeneity test indicated that the ITS2 and 28S regions should be analyzed separately (P=0.01). Heuristic analysis of data for the ITS2 region resulted in 452 trees with identical topologies. For the ITS2

region 221 characters were used. Of these 187 were parsimony informative and 24 parsimony uninformative. For the 28S region 423 characters were used. Of these 101 were parsimony uninformative and 69 parsimony informative. The shortest tree length was 1085 with a RI of 0.749 and CI of 0.489 (Fig. 1). Heuristic analysis of the 28S region resulted in 10068 trees with similar topologies. The shortest tree length was 344 with a RI of 0.755 and CI of 0.692. Comparison of the trees from the different analyses, revealed that in both cases, clades will consist of the same set of species. However, due to the conserved nature of the 28S gene, relationships within the genus could only be resolved into larger clades. Analysis of the ITS2 region was necessary to determine relationships within the *Leptographium* group.

Three distinct clades are obvious from the analysis (fig. 1). The first of these includes *Ophiostoma trinacriforme* (Parker) Harrington, *Leptographium brachiatum* (Kendrick) Wingfield, *L. antibioticum* (Kendrick) Wingfield, *O. brevicolla* Davidson, *L. costaricense* Weber, Spaaij & Wingfield, *L. pineti* Jacobs & Wingfield, *L. pityophilum* Jacobs, Wingfield & Uzunovic, *O. leptographioides* (Davidson) Arx and *L. elegans* Wingfield, Crous & Tzean. Apart from *L. brachiatum* and *L. antibioticum* that are morphologically similar, there is no obvious correlation between morphology and phylogeny within this group.

The second clade included *L. abietinum* (Peck) Wingfield, *L. engelmannii* Davidson, *O. abiocarpum* (Davidson) Harrington, *O. huntii* (Robinson De Hoog & Scheffer, *L. guttulatum* Jacobs & Wingfield, *L. euphyes* Jacobs & Wingfield, *L. alethinum* Jacobs & Wingfield, *L. wingfieldii* Morelet, *O. americanum* Jacobs, Wingfield & Bergdahl, *L. pyrinum* Davidson, *L. hughesii* Jacobs, Wingfield & Harrington, *L. eucalyptophilum* Jacobs, Wingfield & Roux, *L. peucophilum* Jacobs & Wingfield, *O. franke-grosmanniae* (Davidson) De Hoog & Scheffer, *O. penicillatum* (Grosmann) Siemaszko, *O. dryocoetidis* (Kendrick & Molnar) Harrington, *O. robustum* (Robinson-Jeffrey & Davidson) Harrington, *O. crassivaginatum* (Griffin) Harrington and *O. grandifoliae* (Davidson) Harrington.

The third clade has two subgroups. The first of these is comprised of *L. reconditum* Jooste, *O. laricis* Van der Westhuizen *et al.*, *O. europhioides* (Wright & Cain) Solheim and *O. piceaperdum* (Rumbold) Arx and the three varieties of *Leptographium wageneri.* The second group consists of *L. albopini* Wingfield, Harrington & Crous, *O. aureum* (Robinson-Jeffrey) Harrington and *L. lundbergii* Lagerberg & Melin. *Leptographium procerum* and *L. terebrantis* Barras & Perry grouped together separate from the other species (Fig. 1).

Comparison of a sub-set of *Leptographium* spp. with other Ascomycetes using only the 28S region and the heuristic search option in PAUP, produced 288 trees with identical topologies. The group belonging to the Pezizales was used as an outgroup. The shortest tree was 596 with a RI of 0.802 and CI of 0.542 (Fig. 2). The dendogram consisted of three distinct clades. The first clade consisted of species belonging to the Eurotiales and Dothideales. The second clade included species residing in the Microascales, Xylariales, Phyllachorales, Ophiostomatales, Hypocreales and Laboulbeniales and the third clade included the Pezizales. All *Leptographium* spp. grouped closely together within the Ophiostomatales.

Morphological comparison

Analysis of the unweighted morphological characters using the heuristic search option in PAUP, produced 1228 trees with similar topologies with species differing only within clusters. The shortest tree length was 403 with a CI of 0.216 and RI of 0.556 (Fig. 3). The partition homogeneity test on the molecular and morphological data, resulted in a P-value of 0.01, suggesting that the data sets cannot be combined. No correlation could be found between trees produced based on molecular characters (Fig. 1) and those from the analysis of morphological characters (Fig. 3). It was, however, of interest that *O. laricis, O. piceaperdum* and *O. europhioides* clustered together in both the molecular and morphological analysis. The same was true for the three *L. wageneri* varieties.

Weighting of morphological characters produced trees that were not congruent with those produced by the analyses of the unweighted characters (data not shown). Three morphologically important characters, conidiophore length, primary branch type and conidium length to width ration were weighted preferentially. No correlation could be found between any of the weighted trees based on morphology and those based on molecular characters. It is interesting, however, that *Ophiostoma laricis, O. europhioides* and *O. piceaperdum* clustered together as before. The same was true for the three varieties of *L. wageneri.*

The morphological data were also subjected to distance analysis using UPGMA. The resulting cladogram, as in the case of the parsimony analysis, was not congruent with the molecular data. The cladogram consists of four clades and each of these included species of all three groups in the molecular analysis.

DISCUSSION

DNA based analysis of species in *Leptographium* has confirmed some of the taxonomic questions regarding species complexes in this genus. The species that group together in the different clusters based on ribosomal DNA sequence data are morphologically diverse and inhabit different niches. In most cases, no obvious relationships emerged.

Based on analysis of the ribsomal DNA data, *Leptographium abietinum* and *L. engelmannii* were found to be closely related. This confirms the synonymy of *L. engelmannii* with *L. abietinum* as proposed by Zambino and Harrington (1992) and implemented by Jacobs *et al.* (1999). The anamorph of *O. abiocarpum* was not described when the teleomorph of this fungus was described (Davidson, 1966). However, Upadhyay (1981) reported a *Leptographium* state for this species. We have not observed this state in material available to us but, its close relationship with

Leptographium in this study confirms that it fits appropriately with other *Leptographium* spp.

Furthermore, *O. europhioides* appears to be closely related to *O. piceaperdum.* This close relationship confirms the recent synonymy of *O. europhioides* with *O. piceaperdum* proposed by Jacobs, Wingfield & Crous (1999). These species were also found to be closely related to *O. laricis* based on DNA analysis and they are morphologically similar (Van der Westhuizen *et al.,* 1995). Cluster III of the DNA based analysis also includes *L. procerum* and *L. terebrantis.* Although these species are morphologically distinct, they have both been considered similar in terms of disease, they occur on the same hosts and are associated with the same insects (Kendrick, 1962; Barras & Perry, 1971; Wingfield, 1983; Highley & Tattar, 1985; Alexander *et al.,* 1988; Harrington, 1988; Wingfield & Gibbs, 1991). In addition, this cluster includes the three varieties of *L. wageneri.* These varieties are difficult to distinguish morphologically, but can be distinguished based on molecular characters and isozyme analyses (Zambino & Harrington, 1992).

The outcome from the DNA sequence based comparison in this study is similar to that of Zambino and Harrington (1992), which was based on isozyme analyses. Although these authors used a smaller number of species, a significant correlation can be seen between the results of the two studies. Species from the *L. serpens* and *L. lundbergii* cluster (Zambino & Harrington, 1992) corresponded well to cluster III of the ribosomal DNA analysis in this study. *Leptographium albopini* (*L.* sp I), *L. aureum, L. terebrantis, L. lundbergii* and *L. europhioides* formed part of the *L. lundbergii,* together in the isozyme analysis of Zambino and Harrington (1992). This corresponds with the grouping of these species in cluster III of our study. The three varieties of *L. wageneri* clustered together closely in the isozyme study (Zambino and Harrington, 1992) and this was also confirmed in the current study.

The synonymy of *Leptographium engelmannii* with *L. abietinum* as proposed by Zambino and Harrington (1992) and implemented by Jacobs *et al.,* (1999), was confirmed using DNA sequence analysis. These species, together with *O. penicillatum* and *O. abiocarpum* formed part of a separate clade based on isozyme analysis (Zambino & Harrington, 1992). This corresponds to clade II derived from the DNA analysis in the present study. From the DNA sequence data, it appears that *O. abiocarpum* is most closely related to *O. huntii.* However, isozyme analyses on these species placed them in two different groups (Zambino & Harrington, 1992).

Ophiostoma crassivaginatum (Griffin) Harrington clustered separately from the other species based on isozyme comparison (Zambino & Harrington, 1992). This was also reflected in the molecular analysis of this study where *O. crassivaginatum* clustered separately from other *Leptographium* spp in clade II. This species is unlike other species and is characterized by very short, robust conidiophores (Griffin, 1968). *Leptographium procerum* resides in the *L. serpens* group based on isozyme analysis (Zambino & Harrington, 1992). From the DNA sequences data in this study, however, it appears to be more closely related to *L. terebrantis* than to *L. wageneri.* Two species, *O. huntii* and *L. pyrinum,* that reside in cluster II of the DNA sequence study, grouped in the *L. lundbergii* cluster of Zambino & Harrington (1992).

Based on partial sequence of the ITS 1 and 2, as well as the 5.8 S gene, Coetsee (1999), speculated that conidium size might be an indication of phylogeny in some species of *Leptographium.* This was based on the fact that species with long spores clustered separately from those with shorter spores. This observation was also apparent in the current study with all the species having long spores namely, *L. penicillatum, O. americanum* and *O. dryocoetidis,* grouping together. However, this group also contained species with medium sized and small spores and natural relationships do not appear to be reflected by conidium size.

The close relationship between *O. europhioides, O. piceaperdum* and *O. laricis* found in a previous study (Van der Westhuizen *et al.,* 1995; Jacobs *et al.,* 1998), is confirmed here. *Leptographium procerum,* although part of one group in this study, grouped away from the cluster accommodating *O. laricis* and *O. europhioides*. This is consistent with the findings of Coetsee (1999). The only discrepancy between the two studies was with the placement of *Leptographium guttulatum.* Coetsee (1999) found that this species grouped separately from *O. penicillatum.* However, in the current study, *L. guttulatum* groups together with *O. penicillatum* in clade II.

This study has shown that there is no single morphological character that corresponds with phylogeny based on rDNA sequences. However, the rDNA sequence-based data might be reflected in a combination of morphological characters. To test this hypothesis, we coded the morphological characters using a binary code (Viljoen, 1996). Analyses of the unweighted characters produced a dendogram including three clades. Each clade included a group of species from the three main clusters emerging from the sequence analysis. The only groups within the morphological dendrograms that were similar to those emerging from the molecular comparison was the group with *O. piceaperdum, O. europhioides* and *O. laricis* and the cluster including the three varieties of *L. wageneri*. Weighting of different taxonomically important characters did not produce trees that were congruent with results based on rDNA based analysis. This was confirmed through the homogeneity test, which resulted in P values of lower than 0.05. Taxonomically important morphological characters, therefore, cannot be used to infer phylogeny, and only seem to be useful in the identification of these species.

From the molecular data presented in this study, it is apparent that there is no distinction between *Leptographium* spp. that are not known to be associated with a teleomorph, and those that are known to be associated with an *Ophiostoma* state. Earlier researchers have reported a close relationship of morphologically similar genera based on cycloheximide tolerance (Harrington, 1981; Marais, 1996) and the

presence of cellulose in the cell walls of both these genera (Horner *et al.*, 1986; Marais, 1996). This relationship is confirmed in our study. Comparison with other genera of Ascomycetes using partial sequence of the 28S gene, placed all *Leptographium* spp. within the Ophiostomatales, together with *Ophiostoma* spp. *Leptographium* spp. without teleomorphs grouped together with species with known *Ophiostoma* teleomorphs. This implies a strong association between *Leptographium* and *Ophiostoma* as previously suggested (Harrington, 1981; Wingfield, 1993).

Many *Leptographium* spp. are morphologically similar and they are generally difficult to identify (Wingfield, 1993). Thus, many misidentifications have emerged in the scientific literature and these have in some cases also led to misdiagnoses of disease problems. Results of this study have, for the first time provided DNA sequence data for a relatively large set of *Leptographium* spp. We are already using these data to routinely confirm the names of species that are sent to us for identification and we believe that this particular output of this study is especially valuable. Although availability of isolates and cost precluded us from including additional specimens, in future, it will be desirable to extend this initial database. We also hope that similar data sets for other genes will emerge and provide tools for the identification of *Leptographium* spp.

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Table 1. Isolates used in the study.

Species	Ascession number	Isolate number	Host	Origin	Collector
L. abietinum	Not available	CMW 2817	*Picea engelmannii*	USA	T.C. Harrington
L. abicolens	"	CMW 2865	*Abies balsamea*	USA	D.R. Bergdahl
L. albopini	"	CMW 26	*Pinus strobus*	USA	A Lackner
		CMW 2065	*Pinus strobus*	USA	A Lackner
L. antibioticum	"	CMW 2777	*Pinus taeda*	USA	S. Alexander
L. brachiatum	"	CMW 2855	*Picea rubens*	USA	S. Alexander
L. alethinum	"	CMW 3766	*Hylobius abietis* galleries	England	A. Uzunovic
L. costaricense	"	CMW 3041	soil	Costa Rica	P.W. Crous
L. douglasii	"	CMW2078	*Pseudotsuga menziesii*	USA	D. Goheen
L. elegans	"	CMW 2245	*Chaemacyparis* sp.	Japan	M.J. Wingfield
L. eucalyptophilum	"	2.1	*Eucalyptus* spp.	Democratic Republic of Congo	J.Roux
L. euphyes	"	CMW 301			

L. hughesii	"	CMW 4052	*Aquilana* spp.	Vietnam	B. Blanchette
		C930			
L. guttulatum	"	CMW 742	*Pinus sylvestris*	France	M. Morelet
L. lundbergii	"	CMW 30	*Pinus strobus*	New Zealand	M. Dick
L. pineti	"	CMW 3831	*Pinus* sp.	Indonesia	M.J. Wingfield
L. pityophilum	"	CMW 2840	*Pinus nigra*	Italy	S. Frisullo
L. procerum	"	FHM 93-36	*Pinus* sp	USA	M.J. Wingfield
L. pyrinum	"	CMW 169	*Pinus ponderosa*	USA	R.W. Davidson
L. reconditum	"	CMW 15	*Zea mays*	RSA	W. Joost
			rhizosphere		
L. terebrantis	"	CMW 9	*Pinus sylvestris*	Minnesota	M.J. Wingfield
L. wageneri var.	"	CMW 154	*Pseudotsuga*	USA	T.C. Harrington
pseudotsugae			*menziesii*		
L. wageneri var.	"	CMW 402	*Pinus* spp.	USA	T.C. Harrington
wageneri					
L. wingfieldii	"	CMW 2096	*Pinus sylvestris*	France	M.Morelet
O. americanum	"	CMW 495	*Larix decidua*	USA	D.R. Bergdahl
		CMW 2963	*Larix decidua*	USA	D.R. Bergdahl
O. aureum	"	CMW 714	*Pinus contorta* var.	Canada	R.W. Davidson
			latifolia		& T.C.
					Harrington

O. brevicolla	"	CMW 447	*Populus* sp	USA	R.W. Davidson
O. crassivaginatum	"	CMW 90		USA	T. Hinds
O. dryocoetidis	"	CMW 442	*Abies lasiocarpa*	Canada	A.C. Molnar
O. francke-grosmanniae	"	CMW 445	*Quercus* sp.	Germany	H. Francke-Grosmann
O. grandifoliae	"	CMW 703	*Fagus grandifoliae*	USA	R.W. Davidson
O. huntii	"	CMW 2824	*Pinus* sp.	USA	T.C. Harrington
O. laricis	"	CMW 1980	*Larix* sp.	Japan	Y. Yamaoka
O. leptographioides	"	CMW 2803	*Quercus alba*	USA	B. Moss
O. penicillatum	"	CMW 435	*Picea abies*	Germany	
O. piceaperdum	"	CMW 660	*Picea abies*		M. Hallaksella
O. robustum	"	CMW 2805		USA	T. Hinds
O. trinacriforme	"	CMW 670	*Pinus monticola*	Canada	A.K. Parker
O. wageneri (*L. wageneri* var. *ponderosum*)	"	CMW 2821	*Pinus* sp.	USA	T.C. Harrington
Ceratocystis albofundus	AF043605				
Ceratocystis laricicola	AF043600				
Colletotrichum capsici	Z18982				
Colletotrichum	Z18983				

lindemuthianum	
Colletotrichum truncatum	Z18978
Diplodia tumefaciens	AF110816
Epichloe amarillans	U57680
Epichloe baconii	L07138
Epichloe festucae	X62987
Epichloe glyceriae	L07137
Epichloe typhina	L07132
Eupenicillium hirayamae	AF033418
Eupenicillium katangense	AF033458
Eupenicillium reticulosporum	AF033437
Fusarium oxysporum f.sp. *melonis*	M38153
Fusarium solani	L36634
Glomerella cingulata	Z18993
Glomerella graminicola	Z18984
Melanospora fallax	U47834

Melanospora fallax	U17404
Peziza cerea	AF133164
Peziza violacea	AF133171
Sarcosphaera coronaria	AF133172
Scabropezia scabrosa	AF133173
Sphaeropsis sapinea	AF110815
Verticillium lecanii	U17421
Verticillium lecanii	AF049176
Xylaria curta	U47840
Xylaria hypoxylon	U47841

CMW refers to the culture collection of M. J. Wingfield - Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute (FABI), Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, 0002, Republic of South Africa, C refers to the culture collection of T. C. Harrington - Department of Plant Pathology, 351 Bessey Hall, Iowa State University, Ames, Iowa 50011.

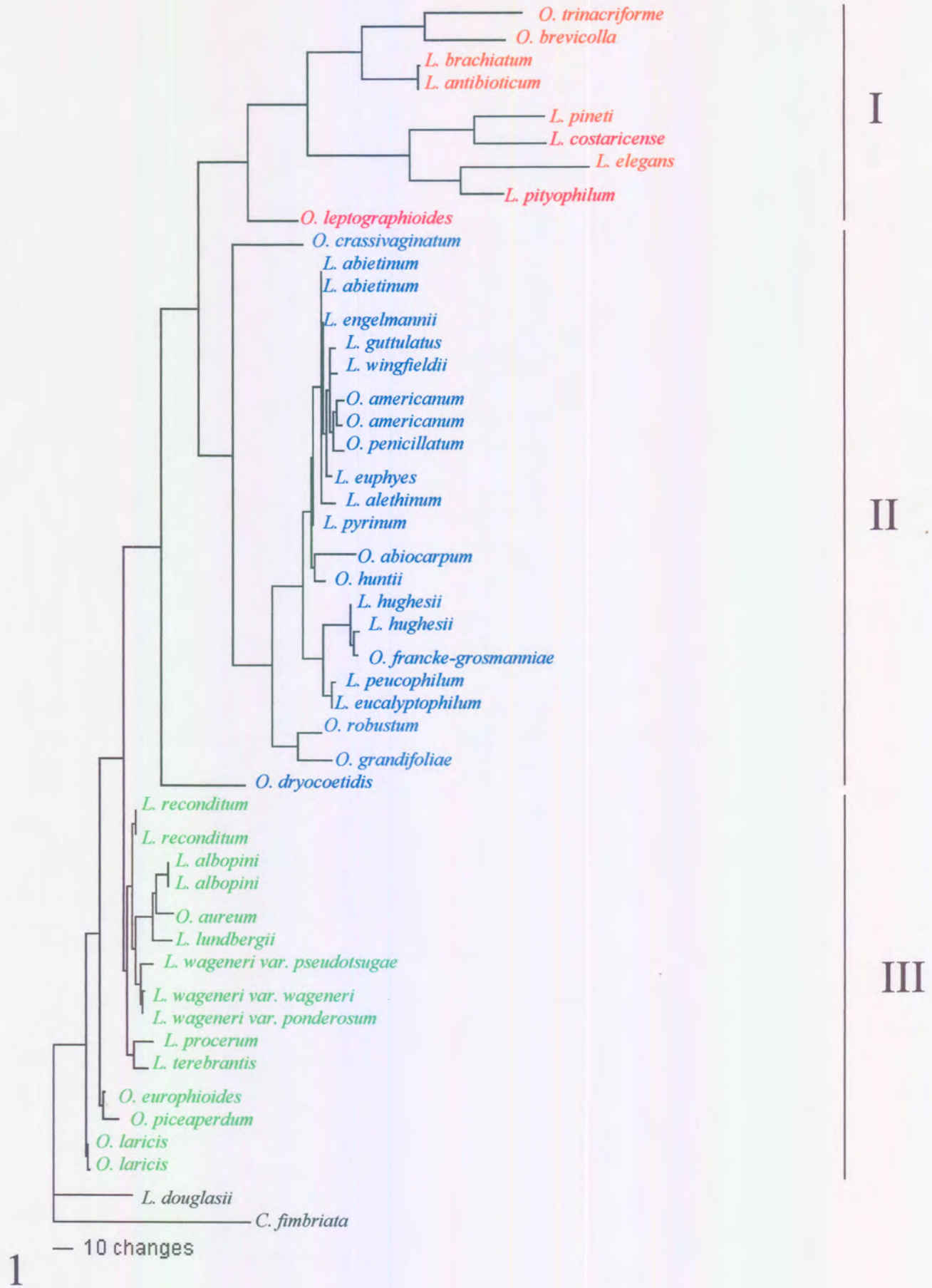
Table 2. Characters used in morphological comparisons

Charater states	1	2	3	4	5	6	7
1. Morphology of hyphae	Constricted at the septa	Not constricted at the septa	-	-	-	-	-
2. Conidiophore length	Less than 100 µm	100 – 200 µm	200 – 400 µm	400 – 600 µm	600 – 800 µm	800 – 1000 µm	1000 – 1500 µm
3. Stipe length	Less than 100 µm	100 – 200 µm	200 – 400 µm	400 – 600 µm	600 – 800 µm	800 – 1000 µm	1000 – 1500 µm
4. Stipe morphology	Not constricted at the septa	Constricted at the septa	-	-	-	-	-
5. Conidiogenous apparatus length	10 – 30 µm	30 – 50 µm	50 – 80 µm	80 – 100 µm	more than a 100 µm	-	-
6. Rhizoids	Present	Absent	-	-	-	-	-
7. Primary branch type	Type A	Type B	Type C	-	-	-	-
8. Number of primary branches	2	2 - 3	3 - 4	4 - 5	more than 5	-	-
9. Primary branch length	Less than 10 µm	10 – 15 µm	15 – 20 µm	more than 20 µm	-	-	-
10. Secondary branch length	Less than 10 µm	10 – 15 µm	15 – 20 µm	more than 20 µm	-	-	-
11. Tertiary branch length	Less than 10 µm	10 – 15 µm	15 – 20 µm	more than 20 µm	To complex to measure	Absent	-

Table 2. (cont.)

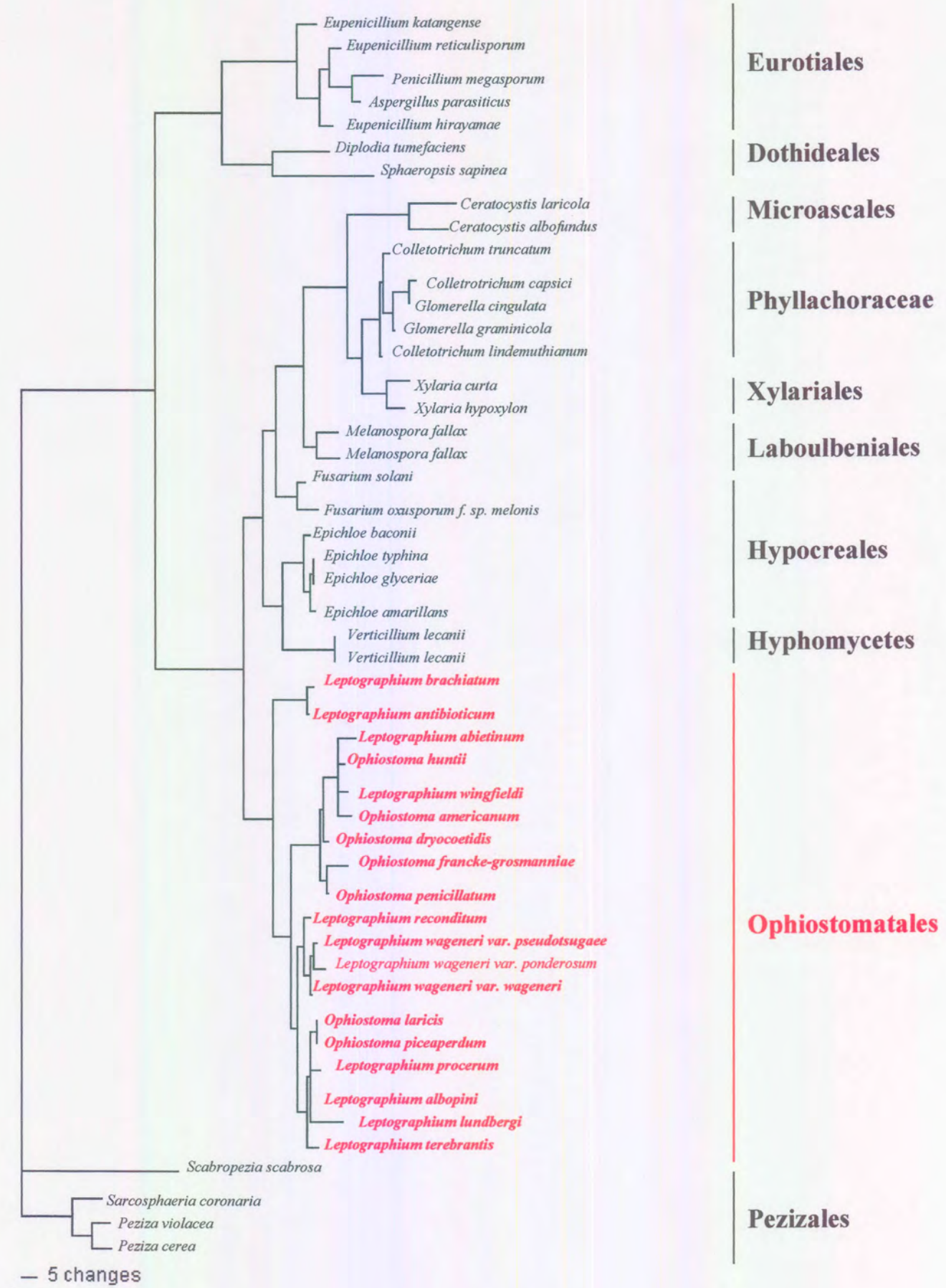
Charater states	1	2	3	4	5	6	7
12. Quaternary branch length	Less than 10 µm	10 – 15 µm	15 – 20 µm	more than 20 µm	Absent	To complex	-
13. Conidiogenous cell length	Less than 10 µm	10 – 15 µm	15 – 20 µm	more than 20 µm	-	-	-
14. Conidium shape	Oblong to obovoid	Obovoid	Distinctly curved	-	-	-	-
15. Conidium length	3 – 5 µm	5 – 7 µm	7 – 10 µm	10 – 12 µm	more than 12 µm	-	-
16. Associated hosts	*Pinus* spp	*Picea* spp.	*Larix* spp.	*Pseudotsuga* spp.	*Abies* spp	Other conifers	Non-conifers
17. Insect association	Associated with insects	Not associated with insects	-	-	-	-	-
18. Optimum growth temperature	15 °C	20 °C	25 °C	30 °C	-	-	-
18. Ratio of conidium length: width	1.5:1	2:1	2.5:1	3:1	4:1	5:1	4:3

Fig. 1. Dendrogram of the DNA based analysis of species in *Leptographium.* 100 trees with identical topologies were obtained through the PAUP analysis using a heuristic search. Trees are rooted to midpoint. The shortest tree length was 1085 with a RI of 0.749 and CI of 0.489.



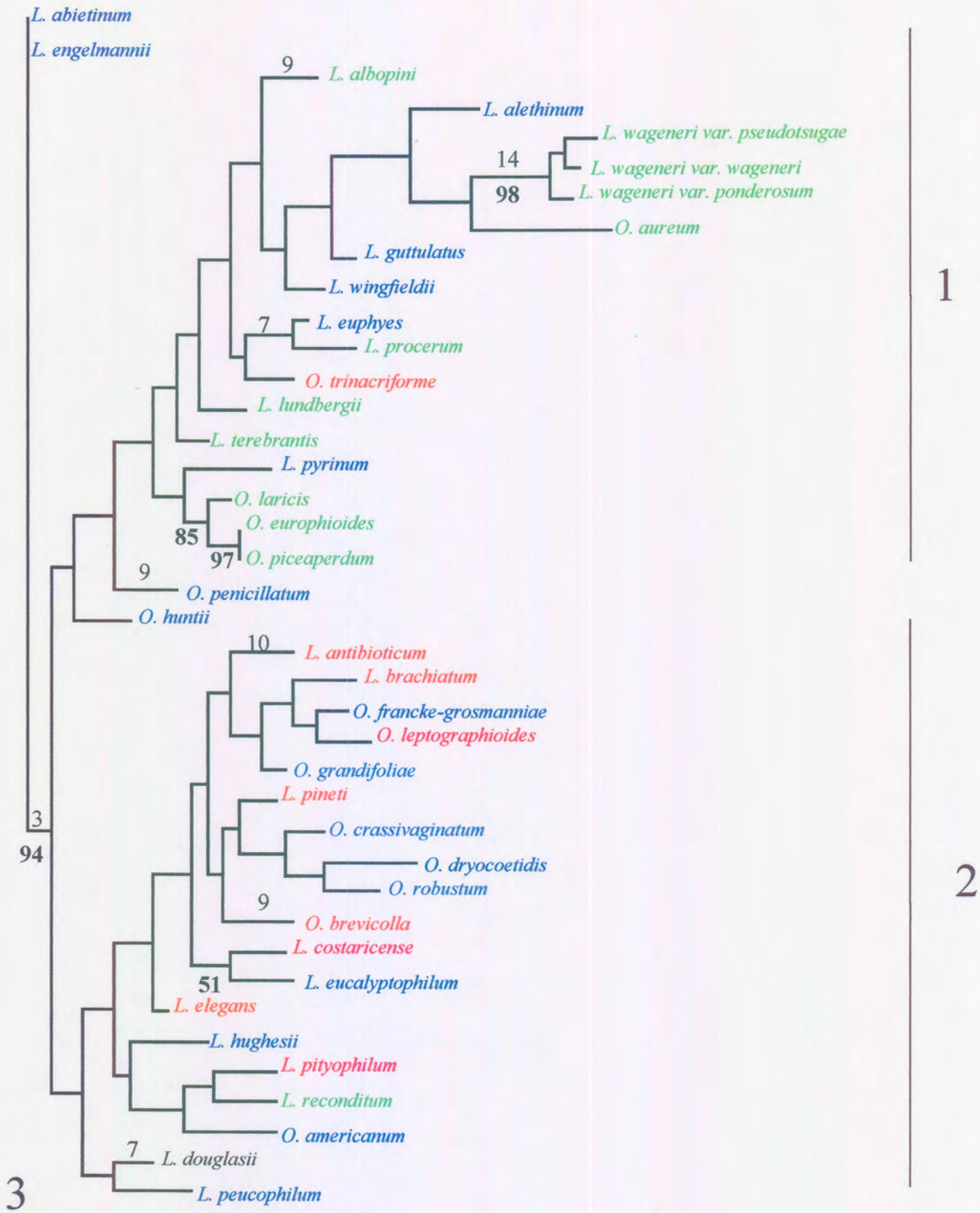
1

Figure 2. Comparison of a sub-set of *Leptographium* spp. to other Ascomycetes using the heuristic search option in PAUP, produced 288 trees with identical topologies . The group belonging to the Pezizales was used as an outgroup. The shortest tree was 596 with a RI of 0.802 and CI of 0.542.



2

Figure 3. Analysis of the unweighted morphological characters using the heuristic search option in PAUP, produced 1228 trees with similar topologies. The shortest tree length was 403 with a CI of 0.216 and RI of 0.556



Chapter 2

Jacobs, K., Wingfield, M.J., Crous, P.W. and Harrington, T.C. (1999). *Leptographium engelmannii,* a synonym of *L. abietinum*, and description of *L. hughesii* sp. nov. Canadian Journal of Botany 76, 1660-1667.

Leptographium engelmannii, a synonym of *L. abietinum*, and description of *L. hughesii* sp. nov.

Leptographium abietinum occurs in North America on various members of the Pinaceae, especially spruce (*Picea* spp.), always in association with bark beetles (Coleoptera: Scolytidae). It is characterized by noticeably curved, clavate conidia. All the isolates were from species of Pinaceae in North America except two isolates examined by Kendrick, originating from *Parashorea plicata* imported to England from Borneo and from *Melia* sp. imported into New Orleans, USA. After examination of the isolate from Borneo and a similar isolate from Vietnam, we have concluded that these do not represent *L. abietinum.* They are described as a new species, *L. hughesii. Leptographium engelmannii,* described from Engelmann spruce in Colorado, USA, is indistinguishable form *L. abietinum* and is considered a synonym of the latter species.

INTRODUCTION

The genus *Leptographium* Lagerb. & Melin includes a number of economically important species associated with root disease and sapstain of timber (Wagener & Mielke, 1961; Harrington, 1988; 1993; Wingfield, Capretti & Mackenzie, 1988; Wingfield, 1993). These fungi are mainly known from conifers, where they are generally associated with bark beetle (Coleoptera: Scolytidae) infestation (Harrington, 1988; 1993; Wingfield, 1993). Some species have also been isolated from non-coniferous hosts, roots and soil (Jooste, 1978; Weber, Spaaij & Wingfield, 1996). Many *Leptographium* spp. are anamorphs of *Ophiostoma*, although some species currently included in the genus lack teleomorphs and are, therefore, of unknown affinity (Jooste, 1978; Harrington, 1987; 1988; Wingfield, 1993; Wingfield, Harrington & Crous, 1994; Wingfield, Crous & Tzean, 1994; Weber *et al.,* 1996).

Leptographium abietinum (Peck) Wingfield occurs on members of the Pinaceae, especially *Picea*, and is associated with species of *Dendroctonus, Hylastes* and *Hylurgops* that infest these trees (Kendrick, 1962; Harrington & Cobb, 1983; Harrington, 1988; Zambino & Harrington, 1992). This species was first described by Peck (1879) as *Sporocybe abietina* Peck and was later transferred to *Periconia* Tode ex Schweinitz by Saccardo (1886). Hughes (1953) recognized the importance of conidium ontogeny as a taxonomic character in anamorphic fungi and established the genus *Verticicladiella* Hughes based on S. *abietina,* which then became known as *Verticicladiella abietina* (Peck) Hughes.

Verticicladiella was thought to be related to *Leptographium* but could be distinguished by differences in the proliferation of the conidiogenous cells. In species ascribed to *Verticicladiella,* proliferation is sympodial, whereas in *Leptographium* species, proliferation is percurrent (Hughes, 1953; Kendrick, 1962). Wingfield (1985) showed that some species in both of these genera displayed apparent sympodial proliferation, which in fact is annelidic with delayed secession

of the conidium, giving it a false sympodial appearance (Van Wyk, Wingfield & Marasas, 1988). He thus reduced *Verticicladiella* to synonymy with *Leptographium.* This included *V. abietina,* which became known as *L. abietinum* (Wingfield, 1985).

The first complete description of *L. abietinum* was provided by Kendrick (1962). Two of the isolates he examined were isolated from hosts other than spruce. One of these hardwood isolates, DAOM 62102 that was used to illustrate the protologue of *L. abietinum* (Kendrick, 1962; p.774), originated from *Parashorea plicata* imported to England from Borneo. The recent availability of an isolate of *Leptographium* from Vietnam that resembles *L. abietinum,* prompted us to re-examine the type material of *L. abietinum,* and the specimen from Borneo that was illustrated by Kendrick (1962).

Leptographium engelmannii, which is known from spruce and associated with the bark beetle *Dendroctonus rufipennis* Kirby (= *D. engelmannii* Hopkins), is also characterized by curved, clavate conidia (Davidson, 1955). Harrington (1988) suggested that *L. engelmannii* and *L. abietinum* might be synonymous, and isozyme analysis (Zambino and Harrington, 1992) supported this synonymy. In the present study, we re-examined *L. abietinum* and compare it to *L. engelmannii* in order to determine whether they could justifiably be maintained as separate species.

MATERIAL AND METHODS

Numerous isolates of *L. abietinum* as well as herbarium specimens of this and other similar species were included in the study. Herbarium isolates examined were *L. abietinum*: slide DAOM 33942, on the bark of spruce, Albany; DAOM 37980, *Picea engelmannii*, A. Molnar, 20 March 1953, Victoria, Canada; DAOM 64328 (DAVFP 11869), *Pseudotsuga menziesii*, C. Cottrell, 20 June 1958,

McGillivray Lake, British Colombia; *L. engelmannii:* US0 422466; *Picea engelmanii,* collected: R. W. Davidson; from Borneo: DAOM 62102, *Parashorea plicata*, Savary, Dec. 1957, Princess Risborough, England, on a ship from Borneo. The herbaria where these isolates are maintained, are as follows: DAOM represents the National Mycological Herbarium, Eastern Cereal and Oilseed Research Centre, William Saunders Building, Agriculture and Agri-food Canada, CEF, Ottawa, K1A 0C6, Canada and USO indicates the National Fungus Collections, Beltsville, Maryland, USA.

Cultures examined included for *L. abietinum*: CMW 2817 (=C699), *Picea engelmannii*, T. C. Harrington, 1993, Dixie Nature Forest, Utah; CMW 276, *Picea engelmannii,* A. Molnar, 1987, Victoria, BC; CMW 3083, *Picea* sp. M.J. Wingfield, August 1994, British Columbia; *L. engelmannii:* CMW 759 (=C29, C713, CO456, RWD971), collected by R. W. Davidson; Vietnam: CMW 4052 (= C930), isolated from the wounds of live *Aquilana* sp., R. A. Blanchette, June, 1996, Phu Quoc Island, southern part of Vietnam. The culture collections where these isolates are maintained, are as follows: CMW represents the culture collection of the Tree Pathology Co-operative Program (Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein, 9301, Republic of South Africa) and C represent the culture collection of T.C. Harrington, Department of Plant Pathology, Iowa State University, Ames, Iowa, 50011, USA

All measurements were made from fungal structures produced in culture on 2 % malt extract agar, (MEA, 20g Biolab malt extract, 20g Biolab agar and 1000 ml distilled water) in 90mm diameter plastic petridishes, containing 20 ml medium . Fungal structures for microscopic examination were mounted on slides in lactophenol. Fifty measurements of each relevant morphological structure were made and ranges and means computed. Herbarium specimens were examined by placing a drop of 1% KOH on the dried material. After five minutes, small portions of fungal material were removed and mounted in lactophenol on glass slides.

Isolates were also examined using scanning electron microscopy (SEM). Small blocks of agar cut from sporulating colonies were fixed in 3% glutaraldehyde and 0.5% osmium tetroxide in a 0.1 M phosphate buffer, dehydrated in a graded acetone series and critical-point dried. Specimens were mounted and coated with gold palladium alloy and examined using a JSM 6400 scanning electron microscope.

The cardinal temperatures for growth of the isolates representing *L. abietinum* (CMW 2817), *L. engelmannii* (CMW 759) and the isolate from Vietnam (CMW 4052) were determined by inoculating eight MEA plates for each isolate at each temperature with a 6 mm diameter colonized agar plug taken from the actively growing margin of fresh colonies. The plates were incubated at temperatures ranging from 5 to 35 °C at 5 °C intervals. Colony diameters were measured after four and eight days, and the size of colonies was computed as an average of eight readings at each respective temperature.

Cycloheximide tolerance of *L. abietinum* (CMW 2817) and *L. engelmannii* (CMW 759) was determined after eight days of growth on 2 % MEA amended with 0.5 mg/ml cycloheximide. The plates were incubated at 25 °C and colony diameters were measured. Cycloheximide tolerance of the Vietnamese isolate (CMW 4052) was determined after eight days of growth on 2 % MEA amended with cycloheximide at 0, 0.05, 0.1, 0.5, 1.0, 2.5 and 5.0 mg/ml after eight days of growth.

RESULTS

The *Leptographium* sp. from Vietnam occurring on *Aquilana* sp. was morphologically identical to the fungus isolated from *Parashorea plicata* from Borneo (DAOM 62102) and illustrated by Kendrick (1962). Another isolate that we examined from hardwood material collected in Malaysia was also morphologically identical to the Borneo material, but this isolate is no longer available. These Southeast Asian isolates have slightly curved conidia and, thus, resemble the type material and other collections of *L. abietinum* from Pinaceae in North America. However, these fungi have very different hosts and geographic distributions, and on close examination they can be distinguished morphologically (Table 1).

Leptographium abietinum is characterized by dark olivaceous colonies on malt extract agar, with conidiophores arising directly from the agar with little aerial mycelium. In contrast, isolates of *Leptographium* sp. from Vietnam, Malaysia and Borneo are characterized by having a dense mat of aerial mycelium covering the colony, with conidiophores occuring in groups on the aerial mycelium and agar surface. The Asian isolates produce rhizoids at the bases of the conidiophore stipes, whereas, these structures are absent or very rarely found in isolates of *L. abietinum* (Fig. 1,2). The conidiophores of the Asian taxon and *L. abietinum* are similar (Fig. 3,4) but those of the Asian taxon are nearly twice as long as those of *L. abietinum* (Table 1). These two taxa can also be differentiated based on conidial morphology. Although the unnamed *Leptographium* sp. has similar curved conidia to *L. abietinum,* most of the conidia are ellipsoidal to obovoid (Fig. 5,6). The Vietnamese isolate also showed an increase in growth rate on 0.1 mg/ml cycloheximide compared to no cycloheximide, with growth inhibition only at higher concentrations of the antibiotic. In contrast, *L. abietinum* had a decreased growth rate when grown on 0.1 mg/ml cycloheximide.

From these observations we conclude that the isolates of the *Leptographium* sp. from Vietnam and Borneo represent an undescribed taxon which is described below.

The type specimen of *L. engelmannii* (USO 422466) was in a poor condition, making comparison with the holotype of *L. abietinum* (DAOM 33942) difficult. A culture of *L. engelmannii* from Davidson's collection, perhaps derived from the holotype, was available for comparison, and the two species appeared morphologically identical. Both species are morphologically similar. In culture, *L. abietinum* and *L. engelmannii* are virtually identical. Both have optimum growth temperatures at 25 °C and both produce cartridge buff to olivaceous (Rayner, 1970) colonies. *Leptographium abietinum* and *L. engelmannii* both tolerate high concentrations of cycloheximide, an indication that they are anamorphs of *Ophiostoma* (de Hoog & Scheffer, 1984; Harrington, 1981). Furthermore, *L. engelmannii* was described from spruce infested with *Dendroctonus rufipennis,* a common bark beetle associate of *L. abietinum* (Harrington, 1988). They also have similar isozyme electromorphs (Zambino & Harrington, 1992). From these data we conclude that *L. engelmannii* is conspecific with *L. abietinum,* and thus their synonymy is proposed below.

Taxonomy

***Leptographium abietinum* (Peck) Wingfield Trans Br. myco. Soc. 85, 92. 1985. Figs. 2,4,6.**

=*Sporocybe abietina* Peck, New York State Museum Report 31, 45. 1879.
=*Periconia abietina* (Peck) Sacc., Sylloge Fungorum, 4, 273. 1886.
=*Verticicladiella abietina* (Peck) Hughes, Can. J. Bot. 31, 653. 1953.

= *Leptographium engelmannii* Davidson, Mycologia 47, 59. 1955.

PLEASE NOTE THAT THE FOLLOWING SPECIES DESCRIPTION SHOULD NOT BE CITED AND ARE PRINTED HERE IN PRELIMINARY FORM. THESE WILL BE FORMALLY PRINTED ELSEWHERE.

***Leptographium hughesii* Jacobs, M.J. Wingfield & Harrington sp. nov.**

Conidiophora evenientia singulatim vel usque ad octona aggregata, exorientia directe ex agaro vel ex mycelio aerio, erecta, macronematosa, mononematosa, 110 - 1120 (medius = 650) µm longitudine, rhizoidaceis structuris praesentibus. Stipites olivaceo-lutei, lenes, cylindracei, simplices, 4-18 septati, 80 - 1130 (medius = 598) µm. Apparatus conidiogenus 27.0 - 92.5 (medius = 60.5) µm longus, massa conidica exclusa, 2 vel 3 (aliquando 4) seriebus ramorum cylindricorum; 2-3 metulae primariae, olivaceo-luteae, leves, cylindricae, aseptatae, 11.0 - 35.5 (medius = 19.0) µm longae et 3.0 µ 6.0 (medius = 4.0) µm latae. Auctus conidii eveniens pariete reponendi causa constructo, holoblastica ontogenie et percurrenti proliferatione et retardata secessione efficiente impressionem falsam proliferationis sympodicae. Conidia hyalina, aseptata, ellipsoidea vel obovoidea, aliquando exique curvata 1.0 - 2.5 x3.0 µ 5.0 (medius = 1.5x4.0) µm.

Colonies with optimal growth at 25 °C on 2 % MEA, reaching 8 mm in diameter after 8 days, with little growth at 5 °C and no growth at 35 °C . Colony olivaceous (21"m) (Rayner, 1970), with lacinate margins. Able to withstand high concentrations of cycloheximide with a 60 % increase in linear growth on 0.1 mg/ml cycloheximide, with a 63 % reduction in growth on 5 mg/ml cycloheximide after 8 days at 20 °C in the dark.

Colony covered in a dense mat of aerial mycelium, hyphae mostly submerged, hyaline, smooth, straight, not constricted at the septa, 1.5 - 6.0 (mean = 3.0) µm

diameter. Conidiophores occurring singly or in groups of up to eight, arising directly from the agar or aerial mycelium, erect, macronematous, mononematous, 110 - 1200 (mean = 650) µm in length, rhizoid-like structures present at the base (fig. 13a). Stipe olive-buff (21'"b), smooth, cylindrical, simple, 4 - 18 septate, 80 - 1130 (mean = 598) µm long (from first basal septum to below primary branches) 3.5 - 7.5 (mean = 5.5) µm wide below primary branches, apical cell not swollen; 5.0 - 12.0 (mean = 8.0) µm wide at base, basal cell slightly swollen (fig. 3, 7, 13b). Conidiogenous apparatus 27.0 - 92.5 (mean = 60.5) long, excluding the conidial mass, with 2 to 3 (occasionally 4) series of cylindrical branches; 2 to 3 primary branches, olive-buff (21'"b), smooth, cylindrical, aseptate, 7.5 - 35.5 (mean = 19.0) µm long and 2.0 - 6.0 (mean = 4.0) µm wide, secondary branches hyaline to olive-buff (21"b), aseptate, 6.0 - 16.0 (mean = 12.0) µm long, 2.0 - 4.0 (mean = 3.0) µm wide; tertiary branches hyaline, aseptate, 4.0 - 13.5 (mean = 8.0) µm long, 1.0 - 3.0 (mean = 2.0) µm wide, quaternary branches aseptate, 6.0 - 8.5 (mean = 8.0) µm long, 1.0 - 2.0 (mean = 1.7) µm wide (fig 8, 13c). Conidiogenous cells discrete, 2 to 4 per branch, tapering slightly from the base to the apex, 8.0 - 18.5 (mean = 12.0) µm long and 1.0 - 2.0 (mean = 1.2) µm wide. Conidium development occurring through replacement wall building with holoblastic ontogeny, percurrent proliferation and delayed secession, giving the false impression of sympodial proliferation (fig. 9-11). Conidia hyaline, aseptate, ellipsoid to obovoid, occasionally slightly curved, 1.0 - 2.5 x 3.0 - 5.0 (mean = 1.5 x 4.0) µm. Basal conidium frill absent (fig. 5, 12, 13d). Conidia accumulating in white, slimy droplets at the apex of conidiogenous apparatus.

SPECIMENS EXAMINED: Herbarium isolates: Holotype: CMW 4052, isolated from the wounds of live *Aquilana* sp., R.A. Blanchette, June, 1996, Phu Quoc Island, southern part of Vietnam; Paratype: DAOM 62102, *Parashorea plicata*, Savary, Dec. 1957, Princess Risborough, England, on a ship from Borneo. Dried specimens and cultures deposited at DAOM and CBS, respectively.

DISCUSSION

Leptographium abietinum is one of the most common fungi occurring on *Picea* spp. infested with *Dendroctonus rufipennis* in North America (Kendrick, 1962; Harrington, 1988; Solheim, 1995). The fungus is characterized by olivaceous colonies and conidiophores ranging in length from 90 to 570 µm and its distinctive narrow, prominently curved conidia. The latter feature was also recognized as taxonomically significant by Kendrick (1962), who unfortunately chose a culture from *Parashorea plicata* in Borneo to represent his revised description and illustration of *Verticicladiella abietinum*.

At present we regard *L. abietinum* as specific to hosts in the Pinaceae, and the species has been isolated from *Picea, Abies, Pinus* and *Pseudotsuga* in North America (Kendrick, 1962; Harrington & Cobb, 1983; Harrington, 1988; Zambino & Harrington, 1992). The fungus has been associated with bark beetles, *Dendroctonus rufipennis, D. pseudotsugae, Hylastes longicollis,* and *Hylurgops planirostris* (Harrington, 1988) and appears to be avirulent or weakly virulent to pine and spruce (Harrington & Cobb, 1983; Reynolds, 1992). *Leptographium engelmannii* from Engelmann spruce in North America is clearly the same fungus, as has been shown in morphological and isozyme comparisons (Zambino & Harrington, 1992). In our opinion, the importance of host, geographical distribution and vectors has been underestimated in the taxonomy of the Ophiostomatoid fungi, including *Ophiostoma* spp., *Ceratocystis* spp. and their anamorphs, including *Leptographium* (Wingfield, 1993).

Leptographium abietinum can easily be distinguished from other species of *Leptographium* based on morphology, particular by its distinct curved conidia. The slightly curved conidia of *L. hughesii* are similar to those of *L. abietinum,* but *L. hughesii* has longer conidiophores, with basal rhizoids and abundant aerial mycelia. The difference in the geographic distribution and host range of these two taxa is noteworthy. All identified *L. hughesii* isolates have been from Southeast

Asia. It appears that *L. abietinum* is restricted to North America. An isolate (C172) from spruce in Scotland is morphologically similar to *L. abietinum,* and it has similar isozyme electromorphs, but can be separated by conidiophore morphology and growth rate (Zambino & Harrington, 1992).

Leptographium hughesii peripherally resembles *L. procerum.* Both these fungi are characterized by long conidiophores (up to 1250 µm) and rhizoids at the bases of the conidiophores. However, these species can easily be distinguished based on the presence of abundant aerial mycelium in colonies of *L. hughesii.* Colonies of *L. procerum* are characterized by submerged mycelia that display concentric zones when grown in culture (Kendrick, 1962). *Leptographium hughesii* is characterized by ellipsoid to obovoid conidia that can be slightly curved in certain cases. In contrast, *L. procerum* are characterized by small (2.5 - 5 µm) obovoid conidia that are never curved. A further difference between these fungi is their host preference. *Leptographium hughesii* is known from non-coniferous hosts, whereas *L. procerum* occurs predominantly on *Pinus* spp. and exclusively on conifers (Kendrick, 1962; Wingfield, 1983; Wingfield *et al.,* 1988). It is best known on white pine (*Pinus strobus* L.), where it has been associated with a disease, known as white pine root decline (Wingfield, 1983; Wingfield *et al.,* 1988; Alexander *et al.*, 1988). No evidence is available to suggest that *L. hughesii* is a pathogen.

Many *Leptographium* spp. have been described from conifers infested with bark beetles (Harrington, 1988; 1993), and *L. hughesii* is unusual in its association with tropical hardwoods. Its vectors have yet to be identified. The cycloheximide tolerance of *L. hughesii* suggests a relationship to *Ophiostoma* (Harrington, 1981), but no perithecia have been associated with this fungus. Recognition of this species confirms the suggestion of Wingfield (1993) that many *Leptographium* spp. remain to be discovered, particularly in poorly studied regions such as Southeast Asia. Future collections in these areas would, therefore, most probably reveal a number of new species in the genus.

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Table 1. Comparison between *Leptographium abietinum*, *L. engelmannii* and *L. hughesii*.

	Species examined		
Characters	***L. abietinum***	***L. engelmannii***	***L. hughesii***
Host	*Abies grandis; Picea engelmannii; P. mariana; P. rubrens; P. ponderosa; Pseudotsuga mensiezii; Pinus contorta; Pinus* spp.	*Picea engelmannii, Pinus contorta*	*Parashorea plicata, Aquilana* sp.
Associated insect	*Dendroctonus Pseudotsuga; D. rufipennis; Hylastes longicollis; Hylurgops planirostris*	*D. rufipennis*	none reported
Distribution	British Columbia, Canada; Eastern and Western USA	Colorado	Borneo, Vietnam
Rhizoids	absent	absent	present
Conidiophore length	96 - 570 µm	100 - 450 µm	240 - 1200 µm
Conidium shape	clavate with curved ends	clavate with curved ends	ellipsoid to obovoid, occasionally curved
Conidium size	4.0 - 7.0 x 1.0 - 2.5 µm	4.0 - 6.5 x 1.0 - 2.5 µm	3.0 - 6.0 x 1.0 - 2.5 µm

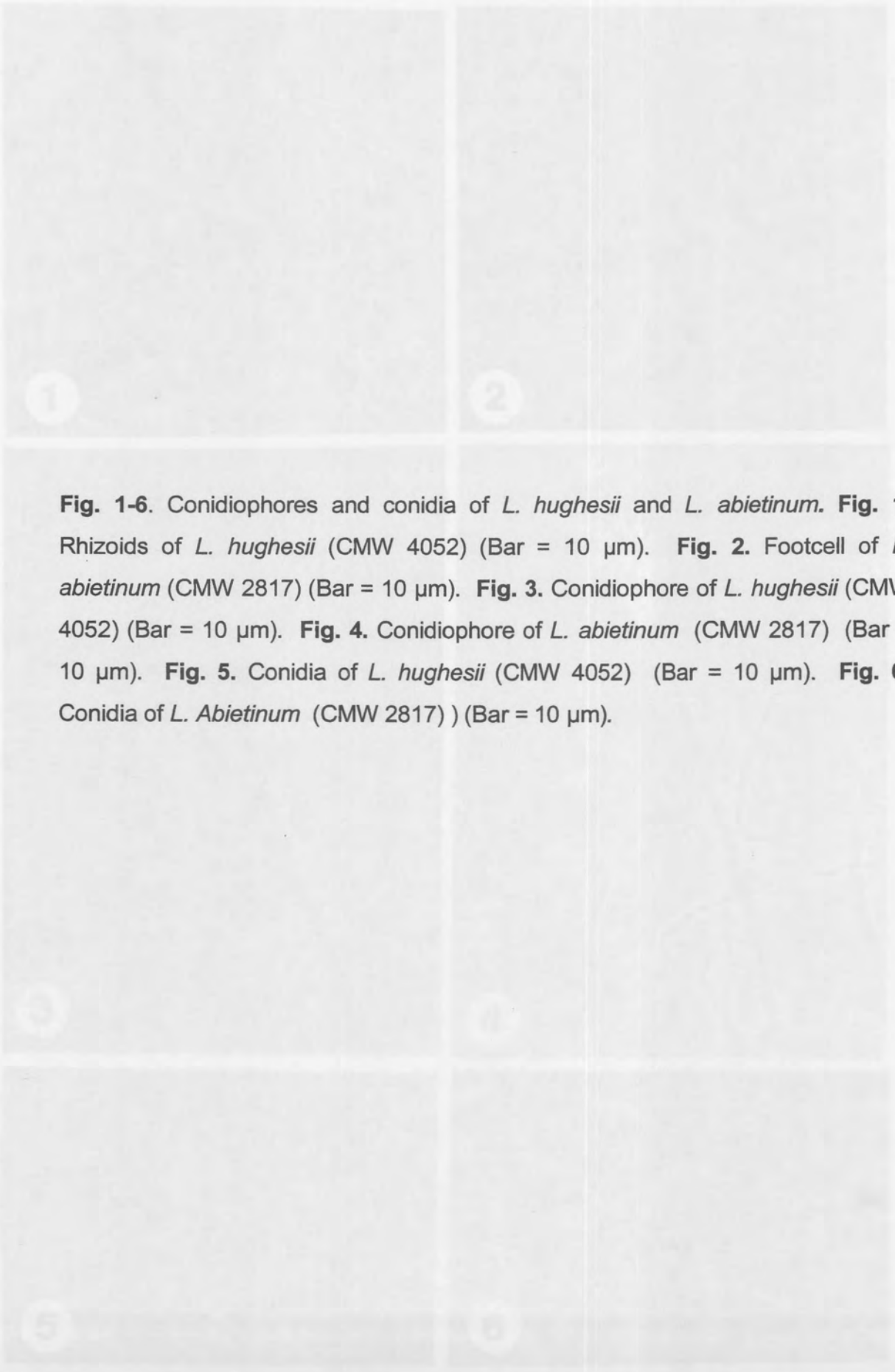
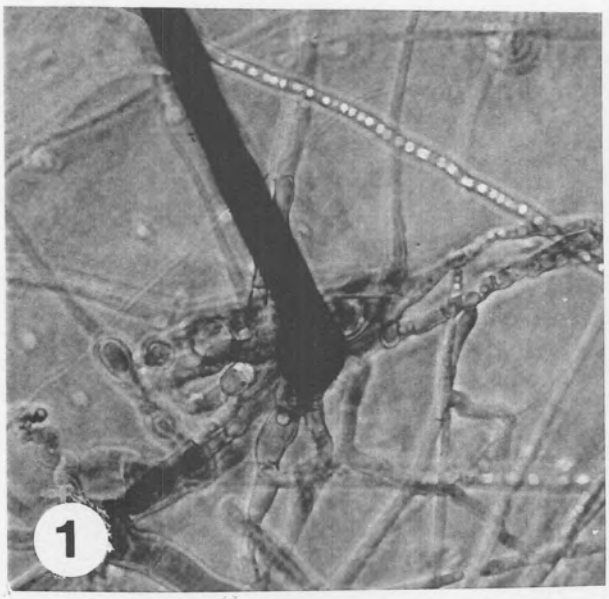
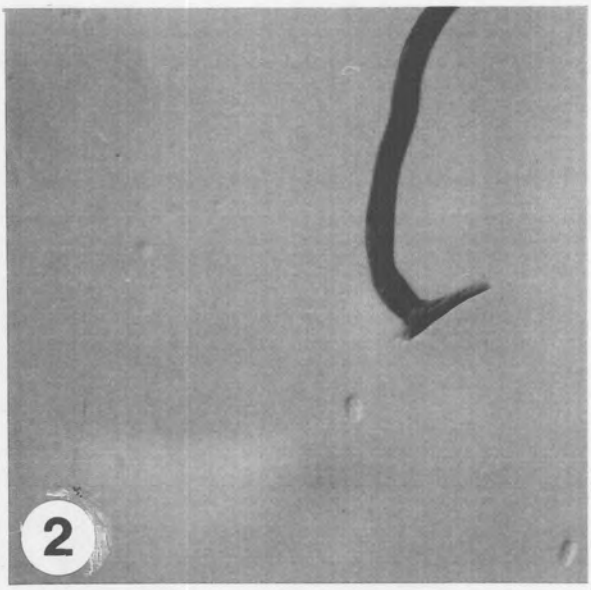
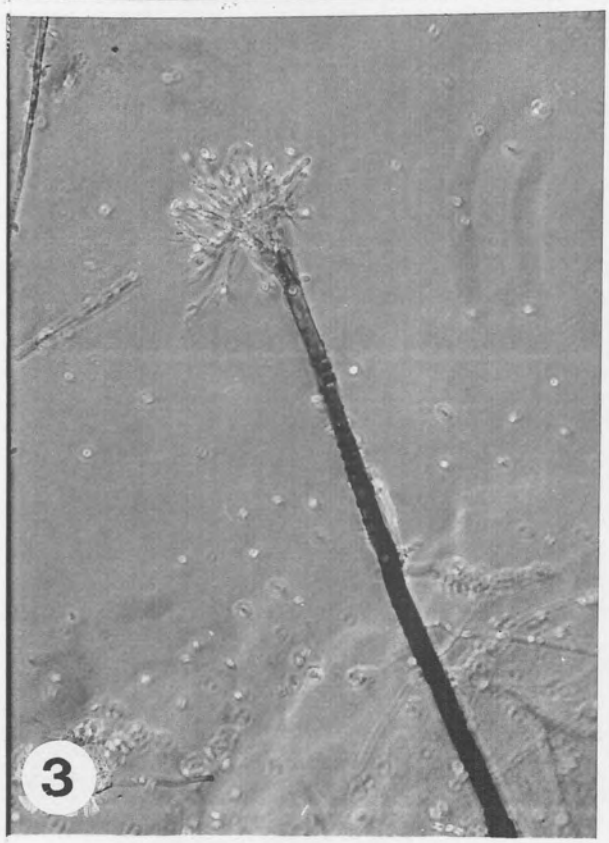
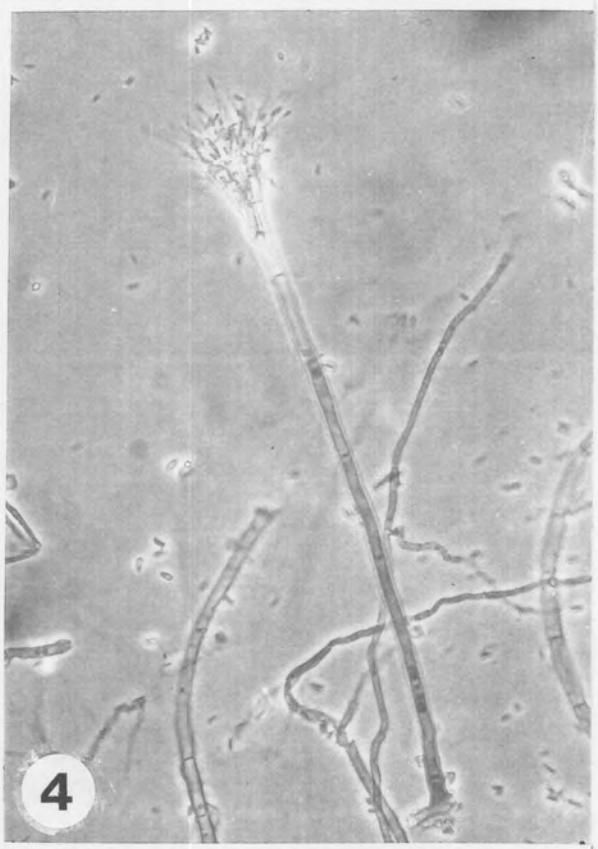


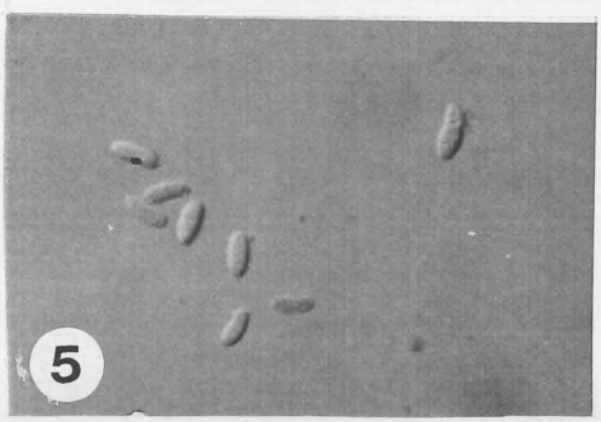
Fig. 1-6. Conidiophores and conidia of *L. hughesii* and *L. abietinum*. **Fig. 1**. Rhizoids of *L. hughesii* (CMW 4052) (Bar = 10 µm). **Fig. 2.** Footcell of *L. abietinum* (CMW 2817) (Bar = 10 µm). **Fig. 3.** Conidiophore of *L. hughesii* (CMW 4052) (Bar = 10 µm). **Fig. 4.** Conidiophore of *L. abietinum* (CMW 2817) (Bar = 10 µm). **Fig. 5.** Conidia of *L. hughesii* (CMW 4052) (Bar = 10 µm). **Fig. 6.** Conidia of *L. Abietinum* (CMW 2817)) (Bar = 10 µm).











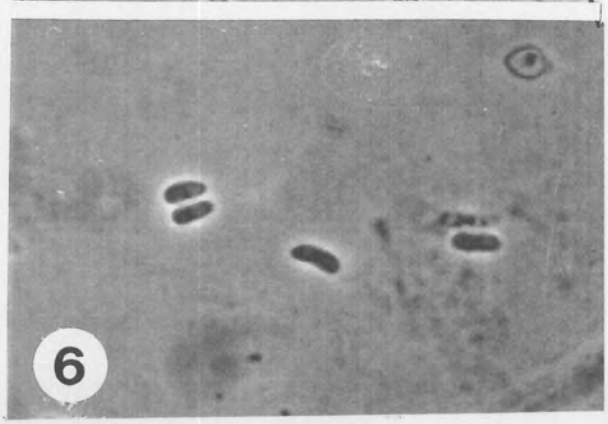
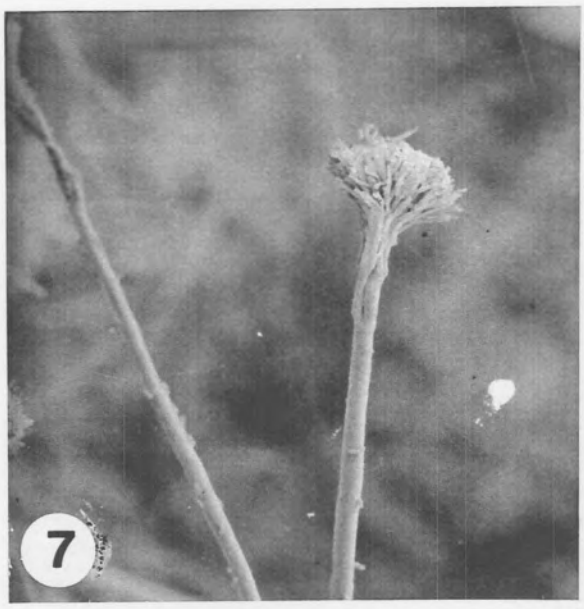
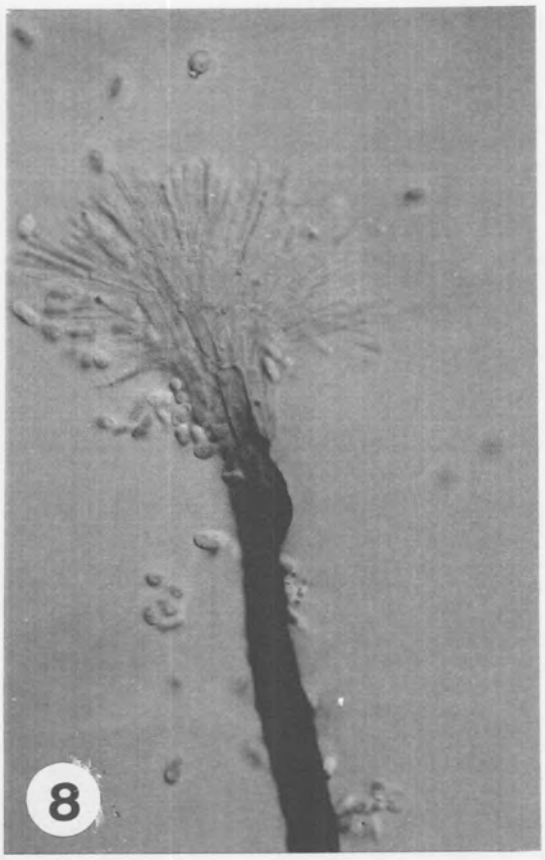
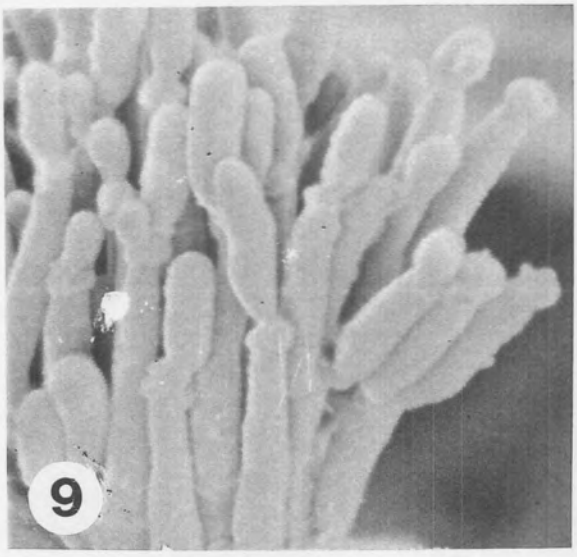
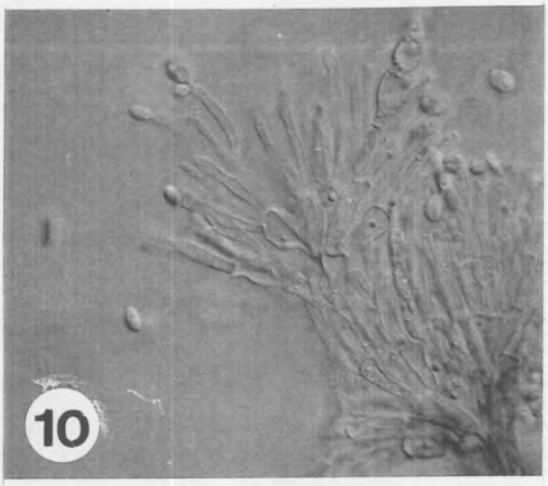


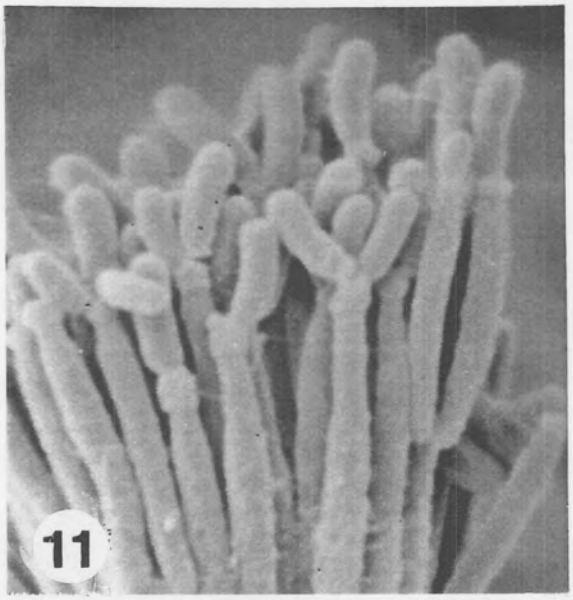
Fig.7-12. Conidiophores and conidia of *Leptographium hughesii* (CMW 4052). **Fig. 7**. Scanning electron micrograph of a conidiophore (Bar = 10 µm). **Fig. 8**. Light micrograph of the conidiogenous apparatus (Bar = 10 µm). **Fig. 9-11**. Light and scanning electron micrographs showing the conidiogenous cells with percurrent proliferation and annelidic conidiogenesis (Bar = 10 µm). **Fig. 12.** Scanning electron micrograph of the conidia (Bar = 10 µm).











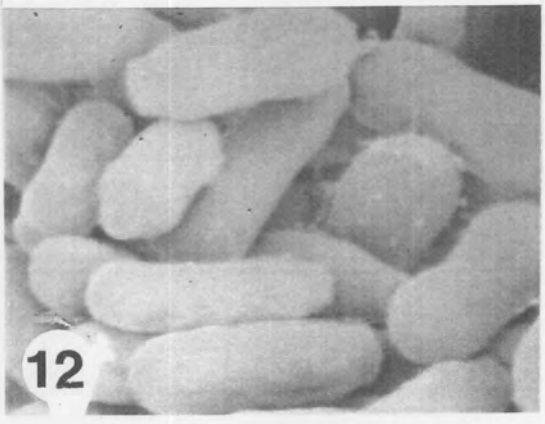
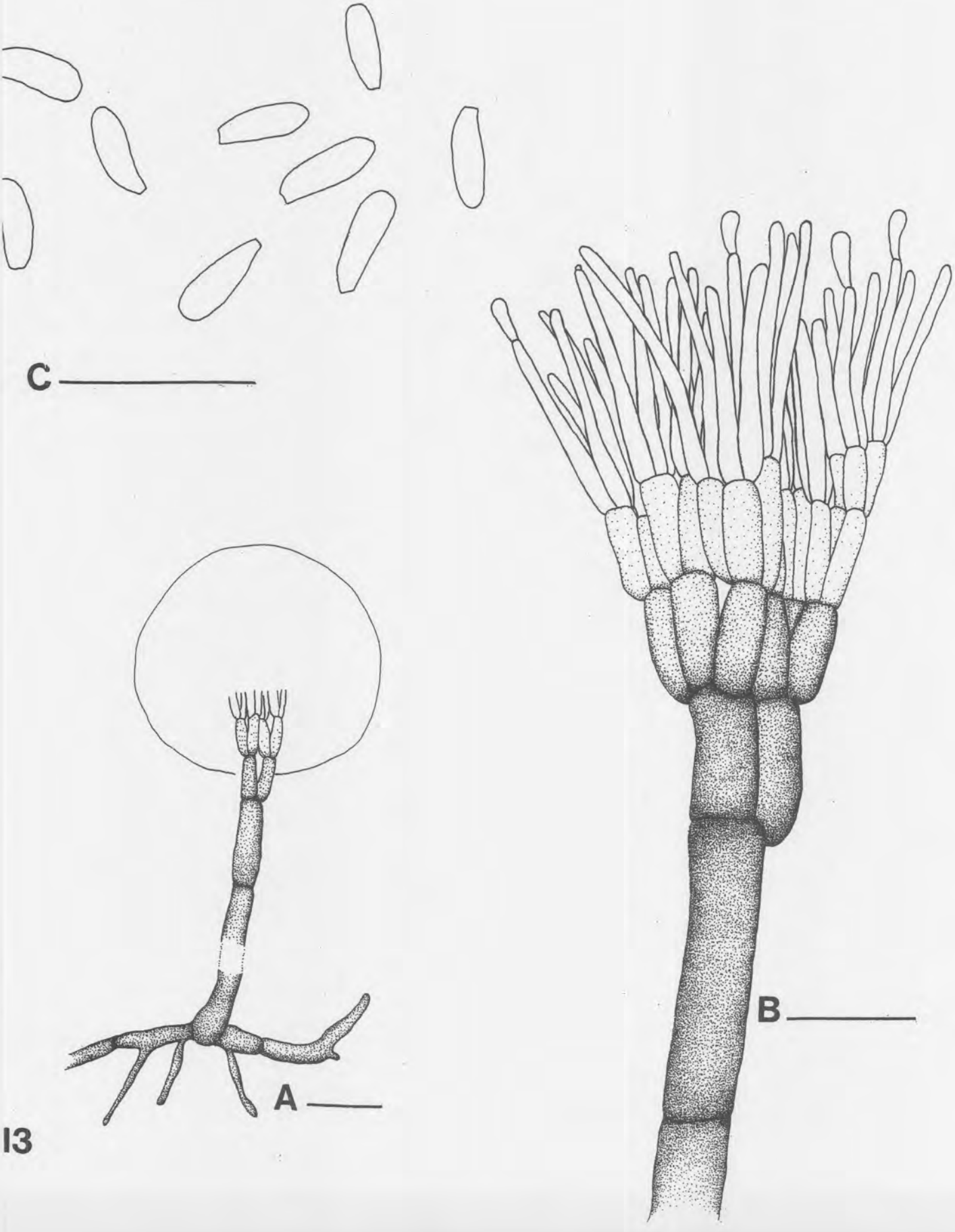


Fig. 13. Conidiophores and conidia of *L. hughesii* (CMW 4052). **Fig. 13a**. Conidiophore with rhizoids present. **Fig 13b**. Conidiophores occurring in groups. **Fig. 13c**. Conidiogenous apparatus (Bar = 10 µm). **Fig. 13d**. Conidia (Bar = 10 µm).



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