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

K. Jacobs, M.J. Wingfield, and B.D. Wingfield

Abstract: Species of *Leptographium* Lagerberg & Melin 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 cause blue-stain in conifers. Few phylogenetic studies have been conducted on *Leptographium* species, and those that have been undertaken have been focused on a small number of species. The objective of this study was to investigate the phylogenetic relationships among 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* Sydow & Sydow. They also provide us with an objective means to confirm the identity of many *Leptographium* species that are difficult to distinguish based on morphological characters.

Key words: *Leptographium*, phylogeny, morphology, *Ophiostoma*, rRNA.

Résumé : Les espèces de *Leptographium* Lagerberg & Melin se caractérisent par des conidiophores mononématiques avec des stipes foncés et des appareils conidiogènes munis de séries de branches complexes. Ces champignons habitent généralement des substrats ligneux, sont associés avec des insectes de l'écorce (Coleoptera : Scotylidae) et causent la tache bleue chez les conifères. Il existe peu d'études phylogénétiques sur les *Leptographium* spp., et celles qui ont été réalisées portent sur un nombre restreint d'espèces. Les auteurs ont donc examiné les relations phylogénétiques entre les espèces de *Leptographium* sur la base de séquences partielles de l'opéron de l'ADN, afin de vérifier si les caractères morphologiques sont congrus avec la phylogénie basée sur l'ADN. Ils ont analysé les caractères morphologiques et les ont comparés avec les résultat des analyses des séquences. Sur la base de l'analyse de ces séquences de l'ADN, les résultats montrent qu'il y a trois groupes au sein du genre *Leptographium*. Cependant, il n'y a pas de congruence entre ces groupes et ceux qui proviennent des caractères morphologiques. Les données de cette étude supportent fortement la relation entre les genres *Leptographium* et *Ophisotoma* Sydow & Sydow. Elles fournissent également un moyen objectif de confirmer l'identité de plusieurs *Leptographium* spp. difficiles à distinguer sur la base des caractères morphologiques.

Mots clés : *Leptographium*, phylogénie, morphologie, *Ophiostoma*, rRNA.

[Traduit par la Rédaction]

Introduction

Species of *Leptographium* Lagerberg & Melin can be recognized by their dark mononematous conidiophores and complex conidiogenous apparatuses. Conidia are produced by annelidic conidium development and are single-celled and hyaline (Kendrick 1962). Some *Leptographium* species are known as anamorphs of *Ophiostoma* Sydow & Sydow species (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 et al. 1986).

Ophiostoma and *Leptographium* species mostly occur on conifers in association with insects, particularly bark beetles (Harrington 1988; Solheim 1986; 1993). Most *Leptographium* species cause blue-stain in the sapwood of lumber. Only one species is well-recognized as a pathogen. This includes the three varieties of *Leptographium wageneri* (Kendrick) Wingfield that cause black stain root disease in the western United States (Wagener and Mielke 1961; Kendrick 1962; Cobb 1988). *Leptographium procerum* (Kendrick) Wingfield has been associated with a root decline disease, primarily in western North America (Harrington and Cobb 1983; Alexander et al. 1988) and *Leptographium*

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Table 1. Cultures used in the study and Genbank accession numbers for sequences included in the analyses.

Species	Accession No.	Isolate No.*	Host or substrate	Origin	Collector
L. abietinum	AF343669	CMW 2817	*Picea engelmannii*	U.S.A.	T.C. Harrington
	AF343670	C699			
L. engelmannii	AF343680	CMW759			
L. abicolens	AF343701	CMW 2865	*Abies balsamea*	U.S.A.	D.R. Bergdahl
L. albopini	AF343695	CMW 26	*Pinus strobus*	U.S.A.	A Lackner
		CMW 2065	*Pinus strobus*	U.S.A.	A Lackner
L. antibioticum	AF343677	CMW 2777	*Pinus taeda*	U.S.A.	S. Alexander
L. brachiatum	AF343676	CMW 2855	*Picea rubens*	U.S.A.	S. Alexander
L. alethinum	AF343685	CMW 3766	*Hylobius abietis* galleries	England	A. Uzunovic
L. costaricense	AF343712	CMW 3041	Soil	Costa Rica	G. Weber
L. douglasii	AF343672	CMW2078	*Pseudotsuga menziesii*	U.S.A.	D. Gobeen
L. elegans	AF343675	CMW 2245	*Chaemacyparis* sp.	Japan	M.J. Wingfield
L. eucalyptophilum	AF343703	CMW 4848	*Eucalyptus* spp.	Democratic Republic of Congo	J. Roux
L. euphyes	AF343686	CMW 301			
L. hughesii	AF343700	CMW 4052/C930	*Aquilana* spp.	Vietnam	B. Blanchette
L. guttulatum	AF343683	CMW 742	*Pinus sylvestris*	France	M. Morelet
L. lundbergii	AF343697	CMW 30	*Pinus strobus*	New Zealand	M. Dick
L. pineti	AF343674	CMW 3831	*Pinus* sp.	Indonesia	M.J. Wingfield
L. pityophilum	AF343679	CMW 2840	*Pinus merbusii*	Italy	S. Frisullo
L. proceram	AF343692	CMW 12	*Pinus* sp.	U.S.A.	M.J. Wingfield
L. pyrinum	AF343689	CMW 169	*Pinus ponderosa*	U.S.A.	R.W. Davidson
L. reconditum	AF343690	CMW 15	*Zea mays* rhizosphere	South Africa	W. Jooste
L. terebrantis	AF343698	CMW 9	*Pinus sylvestris*	Minnesota	M.J. Wingfield
L. wageneri var. *pseudotsugae*	AF343706	CMW 154	*Pseudotsuga menziesii*	U.S.A.	T.C. Harrington
L. wageneri var. *wageneri*	AF343707	CMW 402	*Pinus* sp.	U.S.A.	T.C. Harrington
L. wingfieldii	AF343684	CMW 2096	*Pinus sylvestris*	France	M. Morelet
O. americanum	AF343687	CMW 495	*Larix decidua*	U.S.A.	D.R. Bergdahl
	AF343688	CMW 2963	*Larix decidua*	U.S.A.	D.R. Bergdahl
O. aureum	AF343699	CMW 714	*Pinus contorta* var. *latifolia*	Canada	R.W. Davidson
O. brevicolle	AF343678	CMW 447	*Populus* sp.	U.S.A.	R.W. Davidson
O. crassivaginatum	AF343673	CMW 90		U.S.A.	T. Hinds
O. dryocoetidis	AF343709	CMW 442	*Abies lasiocarpa*	Canada	A.C. Molnar
O. francke-grosmanniae	AF343702	CMW 445	*Quercus* sp.	Germany	H. Francke-Grosmann
O. grandifoliae	AF343711	CMW 703	*Fagus grandifolia*	U.S.A.	R.W. Davidson
O. huntii	AF343682	CMW 2824	*Pinus* sp.	U.S.A.	T.C. Harrington
O. laricis	AF343691	CMW 1980	*Larix* sp.	Japan	Y. Yamaoka
O. leptographioides	AF343710	CMW 2803	*Quercus alba*	U.S.A.	B. Moss
O. penicillatum	AF343704	CMW 435	*Picea abies*	Germany	
O. piceaperdum	AF343694	CMW 660	*Picea abies*		M. Hallaksella
O. robustum	AF343705	CMW 2805		U.S.A.	T. Hinds
O. trinacriforme	AF343671	CMW 670	*Pinus monticola*	Canada	A.K. Parker
O. wageneri (*L. wageneri* var. *ponderosum*)	AF343708	CMW 2821	*Pinus* sp.	U.S.A.	T.C. Harrington
Ceratocystis albofundus	AF043605				

Ceratocystis laricicola	AF043600
Colletotrichum capsici	Z18982
Colletotrichum lindemuthianum	Z18983
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
Scutropezia scabrosa	AF133173
Sphaeropsis sapinea	AF110815
Verticillium lecanii	U17421
Verticillium lecanii	AF049176
Xylaria curta	U47840
Xylaria hypoxylon	U47841

[a]CMW, 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, culture collection of T.C. Harrington, Department of Plant Pathology, 351 Bessey Hall, Iowa State University, Ames, Iowa 50011, U.S.A.

Table 2. Characters of *Leptographium* and *Ophiostoma* anamorphs used in morphological comparisons.

Character states	1	2	3	4
1. Morphology of hyphae	Constricted at the septa	Not constricted at the septa	—	—
2. Conidiophore length (μm)	<100	100–200	200–400	400–600
3. Stipe length (μm)	<100	100–200	200–400	400–600
4. Stipe morphology	Not constricted at the septa	Constricted at the septa	—	—
5. Conidiogenous apparatus length (μm)	10–30	30–50	50–80	80–100
6. Rhizoids	Present	Absent	—	—
7. Primary branch type	Type A	Type B	Type C	—
8. No. of primary branches	2	2–3	3–4	4–5
9. Primary branch length (μm)	<10	10–15	15–20	>20
10. Secondary branch length (μm)	<10	10–15	15–20	>20
11. Tertiary branch length (μm)	<10	10–15	15–20	>20
12. Quaternary branch length (μm)	<10	10–15	15–20	>20
13. Conidiogenous cell length (μm)	<10	10–15	15–20	>20
14. Conidium shape	Oblong to obovoid	Obovoid	Distinctly curved	—
15. Conidium length (μm)	3–5	5–7	7–10	10–12
16. Associated hosts	*Pinus* spp.	*Picea* spp.	*Larix* spp.	*Pseudotsuga* spp.
17. Insect association	Associated with insects	Not associated with insects	—	—
18. Optimum growth temperature (°C)	15	20	25	30
19. Ratio of conidium length:width	1.5:1	2:1	2.5:1	3:1

serpens (Goidanich) Arx with a root disease in pines in South Africa and Italy, but the role of these fungi in disease remains unclear (Lorenzini and Gambogi 1976; Wingfield and Knox-Davies 1980; Wingfield et al. 1988).

Several new species have been described in *Leptographium* in recent years (Wingfield et al. 1994; Van der Westhuizen et al. 1995; Jacobs et al. 1997, 1998, 1999*b*; Webber et al. 1999). Some of these are unusual, because they are associated with niches such as soil and nonconiferous hosts. Several have also been described from tropical areas, which is unusual for *Leptographium* species (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 among some *Leptographium* species. 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* species, Jacobs et al. (2001) 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 et al. 1997; Ward and Adams 1998; Myburg et al. 1999; Witthuhn et al. 1999). This is especially true for morphologically similar taxa (Glenn et al. 1996; Wingfield et al. 1996; Dupont et al. 1998; O'Donnell et al. 1998; Chen et al. 1999; Myburg et al. 1999; Witthuhn et al. 1998). 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 et al. 1993*a*, 1993*b*; Wingfield et al. 1999). The majority of these studies have made use of the ITS and 5.8S ribosomal RNA operon sequences. However, the large subunit of the ribosomal gene (28S) is sufficiently conserved to allow determination of relationships among genera but is also sufficiently sensitive to distinguish relationships among species in many cases (Gaudet et al. 1989; Yamada and Kawasaki 1989; Yamada et al. 1989; Guého et al. 1989; Kurtzman and Robnett 1991; Peterson and Kurtzman 1991; Hausner et al. 1993*b*; Vilgalys and Sun 1994; Wingfield et al. 1994*a*).

Various authors have attempted to correlate morphological characters and DNA sequence phylogeny, with various levels of success. Berbee and Taylor (1992) concluded that some morphological characters can be misleading and may not be a reflection of true relationships in ascomycetes. This was also the case with Hausner et al. (1992) and Wingfield et al. (1994*a*), who found that relationships inferred from ascospore shape were incongruent 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 et al. 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 morphological data for *Lentinus* species in a manner similar to that used for sequence data and analyzed these data as they would sequence data. From the results, they determined whether relationships based on morphology corresponded to those determined through molecular phylogeny. A similar approach was followed by Viljoen (1996) who studied the phylogeny of *Ceratocystis* s.l. based on morphological characters. Patterson et al. (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 objective of this study was to determine the phylogenetic relationships of species of *Leptographium* through comparison of the partial sequence of the ITS2 (internal transcribed spacer region) and 28S ribosomal RNA operon. A selected set of isolates was used to determine the placement of *Leptographium* in a larger group of Ascomycetes.

5	6	7
—	—	—
600–800	800–1000	1000–1500
600–800	800–1000	1000–1500
—	—	—
>100	—	—
—	—	—
—	—	—
>5	—	—
—	—	—
—	—	—
Too complex to measure	Absent	—
Absent	Too complex to measure	—
—	—	—
—	—	—
>12	—	—
Abies spp.	Other conifers	Nonconifers
—	—	—
—	—	—
4:1	5:1	4:3

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 the selected morphological characters are congruent with those generated based on DNA sequence data.

Materials and methods

Molecular comparisons

Representative isolates were selected for known *Leptographium* species (Table 1). These cultures represent the majority of the described *Leptographium* species. Species not included in the study are *Leptographium aenigmaticum* Jacobs, Wingfield, & Yamaoka; *Leptographium neomexicanum* Wingfield, Harrington, & Crous; and *L. serpens*. These species were omitted because we failed to amplify the DNA of the desired region despite repeated attempts. One possible explanation is that there might be variation in the annealing sites of the chosen primers. Where possible, and within the limitations of our budget, more than one isolate per species was included.

The DNA extractions were performed using a modification of the technique described by Raeder and Broda (1985). Genomic DNA was extracted from 2-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 mL extraction buffer (1% cetyltrimethylammonium bromide (CTAB), 200 mM Tris-HCl (pH 8.0), 25 mM EDTA, 250 mM NaCl 1.5% SDS). This was then incubated in a 60°C waterbath for 1 h. Proteins were removed with phenol and chloroform (1:1), followed by a series of chloroform extractions, until the interface was clean. The DNA was precipitated with cold 100% ethanol and left for 2 h at –20°C. This solution was then centrifuged at 13 000 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 ultraviolet light.

The ITS2 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, U.K.). 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 10× PCR buffer (Boehringer Mannheim, Germany), 5 mM $MgCl_2$ (Boehringer Mannheim, Germany), 10 mM dNTPs, 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, and 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 an ABI 377 automated sequencer using the Thermo Sequenase dye terminator cycle sequencing premix kit (Perkin Elmer Applied Biosystems). Sequence data were edited using Sequence Navigator (Perkin Elmer Applied Biosystems) and aligned using the alignment algorithm, CLUSTAL. Manual adjustment of the alignments was done in PAUP* (version 4.0). Analyses were performed in PAUP* (version 4.0) (Phylogenetic Analysis Using Parsimony *and other methods) (Swofford 1999). Gaps were treated as missing data. Parsimony analyses were performed using the heuristic search option (TBR, tree bisection–reconnection). Bootstrap support was determined on 1000 bootstrap replicates, using the "fast"-bootstrap option. Selected *Leptographium* rRNA operon DNA sequences were also compared with other genera of the Ascomycetes. Sequences for other taxa for comparisons were obtained from Genbank (Table 1).

A Templeton nonparametric Wilcoxon signed ranked test was performed on the different data sets (Kellogg et al. 1996; O'Donnell 2000). This test gives an indication of whether data sets can be combined or analysed separately. The tests were performed to determine whether ITS2 sequence data, LSU sequence data, and morphological data were appropriate for combination.

Morphological comparison

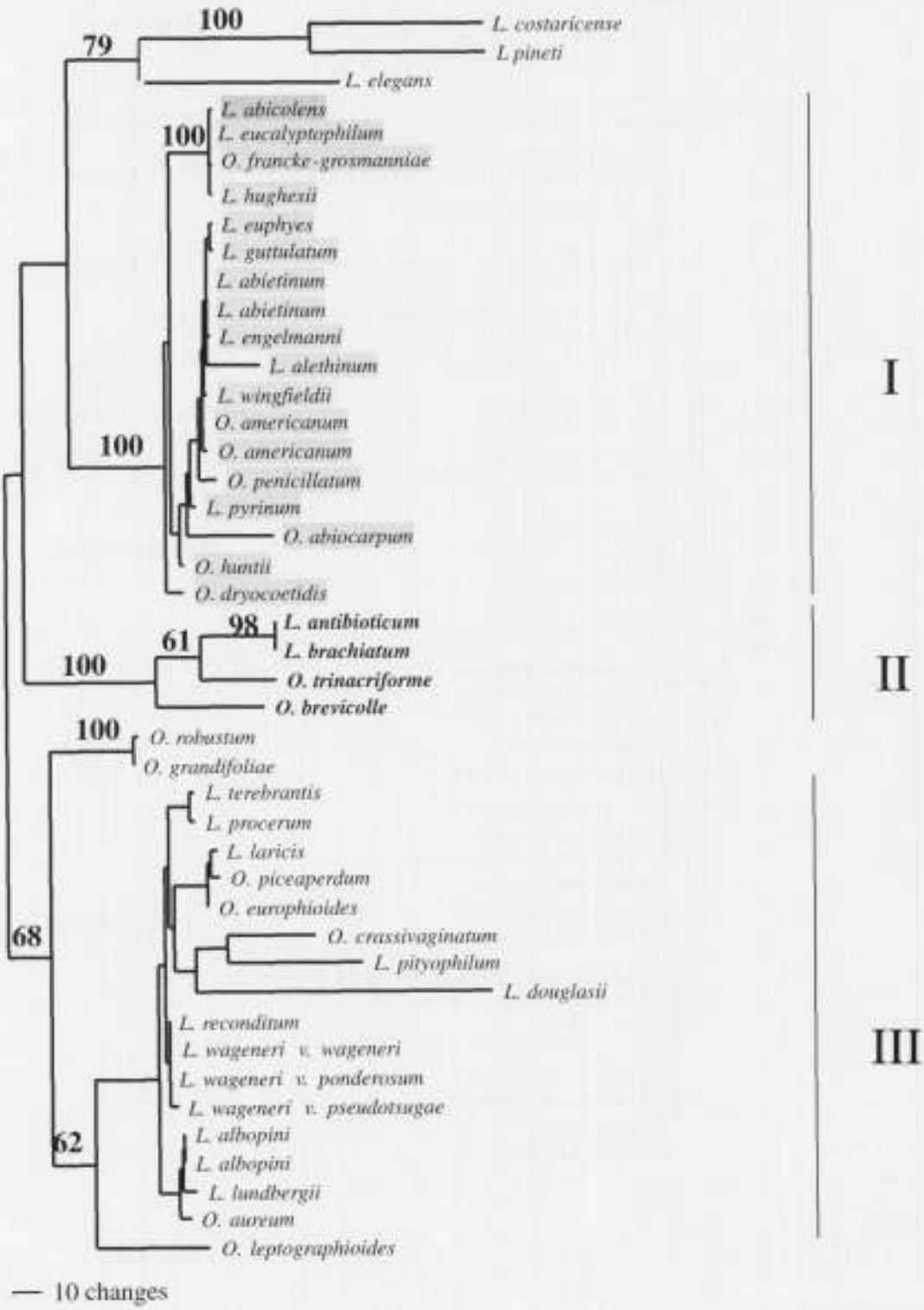
The data used for comparison were obtained from a morphological study of all described species in *Leptographium* (Jacobs and Wingfield 2001). Representative isolates of each species were grown on 2% malt extract agar (MEA) (Table 1). These were compared with the original description of each species 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 were computed.

Nineteen different characters were used in the comparison. Only about half of all described *Leptographium* species have been connected to a teleomorph (Harrington 1988; Wingfield 1993), 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 multistate character and species were coded according to their range. Similarly, there are three forms of primary branch arrangement in *Leptographium*,

Fig 1. Data matrix of morphological characters coded as multistate characters.

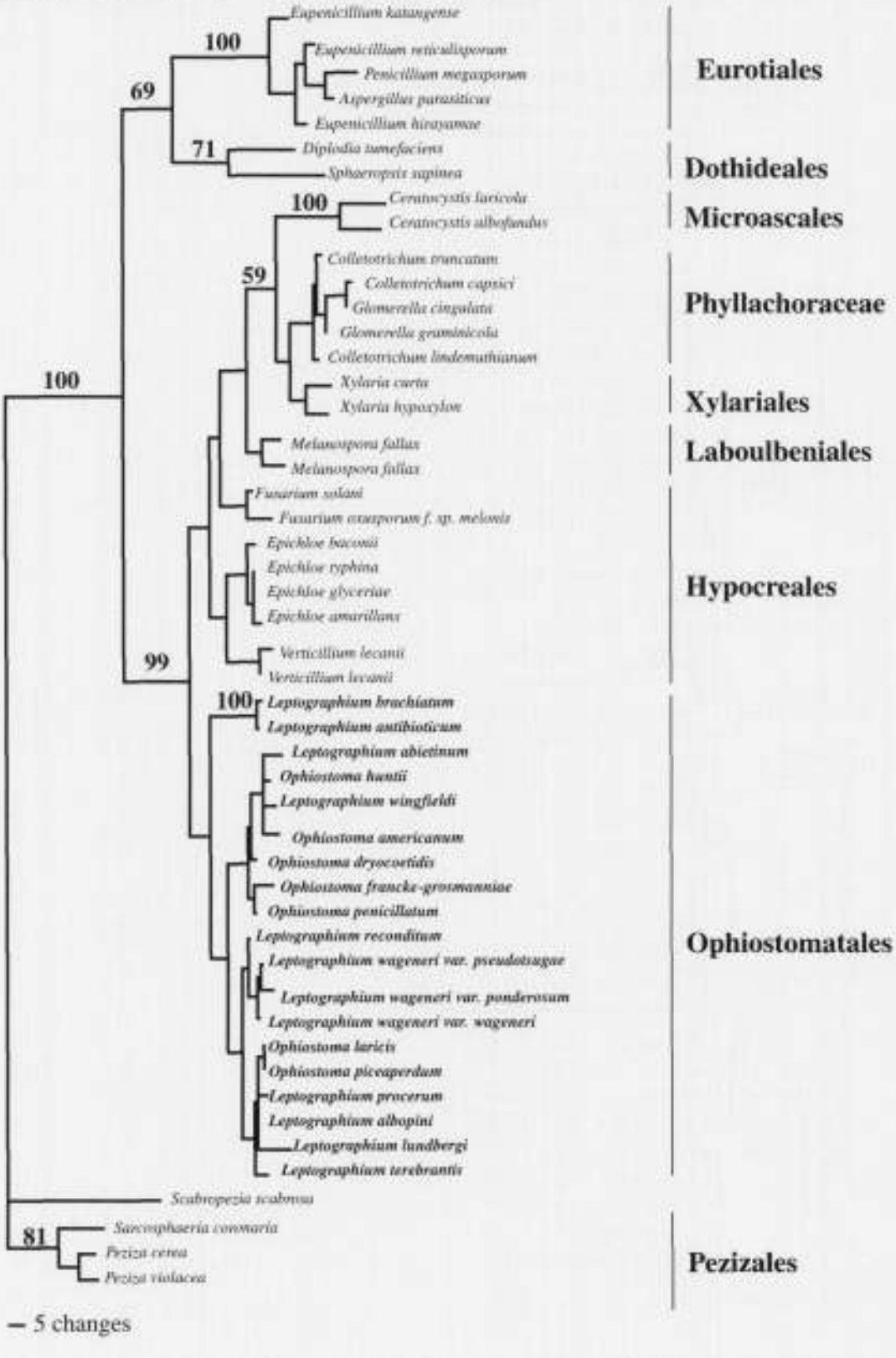
L. abietinum	0212340001234000101234002023120001234001230001200001200000234003120001204000020030003000
L. englmannii	0212340001234000101234002023120001234001230001200001200000234003120001204000020030003000
L. albopini	1002345600234560100234502020123451234561234000230001230000234100120001000000002003000300
L. antibioticum	0202300001230000101230010020123401200001200001200000000501200100100001000560100004003000
L. brachiatum	0212000001200000101200010100100001234001200000200000000501230100100000204000100030003000
L. alethinum	0200045670004567100230002020123000000056023450023000123000023402012300000020002020000040
L. costaricense	0202345000234000101200002020123000230001000001000000000501230020100000000000710003002000
L. douglassi	1212340001234000101230010020120001234001230001200001000001234020120000004000020200003000
L. elegans	0202340001230000101234002020120000234001200001200000000500234100100000000006010003000300
L. eucalyptophilum	0202340000234000100230002020120000234501200001000000000501200100023000000007100004020000
L. euphyes	1202300000230000100234010020120000234561230001200001200000230100120001000000002003002000
L. hughesii	0202345671234567101234010020120001234561230001200001000001230103100000000007100030003000
L. guttulatus	0200345000234500100034502020123000034560034500234001234000234100123001200000020030000400
L. lundbergii	1202345001234000100234502020120000234561234501234000000500234100100001230000020030000007
L. peucophylum	0200340000234000100234510020120001234001230001200001200001230020120000020000002100002000
L. piniti	0202300001200000100230002020120000023000120000120000000050120002010000100000000200300300
L. pityophilum	0202345000234000100234002003123400234001230001200001200000230020120001000000010020002000
L. procerum	1202345000234500101234010020120000234561200001200001200000230100100001204500020030020000
L. pyrinum	0202300001230000100004510020123000234560234500234000234000234100023401000000002003010000
L. reconditum	0202345000234500100234010003123401234001230001200000000501234100100000000007100200003000
L. terebrantis	1202340001234000100234502020120000234561234501234001000001234020123001004000020030020000
L. wagvpseud	1200005670004560100034502003123400234561234501230000000501200100123000004000020200003000
L. wagvwag	0200005670004560100034502003123400234561234501230000000501230100123001030500020200003000
L. wingfieldii	1202345000234500100034502020123000034001234001234001230001234100120001000000020030020000
O. americanum	0202345000234500101230002100100001230001230001200001230001234100123450030000020200000500
O. aureum	1002345671234500100234502020120000034560000060000500000061234100023401000000002020000300
O. brevicollis	0202300001230000101200002100100001230001200000000006000050120010012000000000702000400300
O. crassivaginatum	1212000001000000021230002020120001234001230001000000000501200100123001200000020030003000
O. dryocoetidis	1012300001200000101230002020120001230001200001200001200000230100003450000500020030000006
O. franckegrosm	0212000001200000101200010020120001200001200000000006000050120010010000000000702003002000
O. grandifoliae	0212300001230000101200010020120001200001200001000000000500230020100000000007100030003000
O. huntii	0202345000234500101200002020120001234501230001230001200000234020123001200000020030003000
O. laricis	0202300001200000100034502020120000234500234501234001230000234020123000030000020030003000
O. leptographiodes	0212300001200000101200010020120001230001200000000006000050120010012340000000710000400040
O. penicillatum	0202340001230000100234002020123400234501234001230001200000234003123000200500020004000 4000
O. piceaperdum	0202300001200000100034502020120000034560234001234001230000234020123001204000020030003000
O. europhioides	0202300001200000100034502020120000034560234001234001230000234020123001204000020030003000
O. robustum	0212000001000000101230002020120001230451230000000060000501234100003451000000020030100000
O. trinacriforme	1202305001234000100234002020120000234560234001230001200001234100120001000000100030003000
O. wagvpond	0200005600004560100034002003123401234561234001200000000501230100123001000000020200003000

Fig. 2. One of the most parsimonious trees of the DNA based analysis of species in *Leptographium*; 285 trees with similar topologies were obtained through the PAUP analysis using a heuristic search. Trees are rooted to the midpoint. The shortest tree length was 901 steps with a retention index (RI) of 0.832 and consistency index (CI) of 0.628. Bootstrap values are indicated in bold on the branches. The different fonts correspond to the three major groups derived from analysis of the molecular data and is indicated as such to simplify comparison with the analysis of the morphological characters in Fig. 4.



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 is two 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 be defined broadly in

Fig. 3. Comparison of a subset of *Leptographium* species to other Ascomycetes using the heuristic search option in PAUP produced 288 trees with similar topologies. The group belonging to the Pezizales was used as an outgroup. The strict consensus tree is presented here. The shortest tree was 596 steps with a RI of 0.802 and CI of 0.542.



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 multistate coding system (Table 2). Characters with ranges were polarized by defining them as ordered characters in the PAUP analysis. Characters that did not represent ranges were defined as unordered characters. A matrix was compiled for the data set, and data were analyzed using parsimony analysis, as well as distance analysis (UPGMA) using the PAUP* version 4 program (Swofford 1999) (Fig. 1). 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 100. A fast-bootstrap analysis (1000 replicates) was done to calculate bootstrap support. No outgroup taxa were used and the trees were rooted to midpoint.

Results

Molecular comparison

A single amplified fragment of 950 base pairs (bp) was obtained for all the isolates using the primers ITS3 and LR3. Of these, 648 bp were used for comparison. This region included the last 10 bp of the 5.8S gene, the whole ITS2 region, and the first 416 bp of the 28S gene. Results from the Templeton nonparametric Wilcoxon signed ranked test indicated that the ITS2 and 28S regions should be analyzed separately ($P = 0.0001$). Heuristic analysis of data for the ITS2 region resulted in 285 trees with similar topologies. For the ITS2 region, 240 characters were used. Of these, 180 were parsimony informative, 43 were parsimony uninformative, and 17 were constant. The shortest tree length was 901 steps with a retention index (RI) of 0.832 and a consistency index (CI) of 0.628 (Fig. 2). The 28S region was analysed separately. For the 28S region, 416 characters were used. Of these, 51 were parsimony informative, 84 were parsimony uninformative, and 281 characters were constant. Heuristic analysis of the 28S region resulted in 10 068 trees with similar topologies. The shortest tree length was 344 steps with a RI of 0.755 and CI of 0.692. Comparison of the trees from the different analyses revealed that, in both cases, groups consisted of the same sets of species. However, because of the conserved nature of the 28S gene, relationships within the genus could only be resolved into larger groups. Analysis of the ITS2 region was necessary to determine relationships within the *Leptographium* group.

Three distinct groups are obvious from the analysis (Fig. 2). The first of these includes *Leptographium abietinum* (Peck) Wingfield; *Leptographium engelmannii* Davidson; *Ophiostoma abiocarpum* (Davidson) Harrington; *Ophiostoma huntii* (Robinson-Jeffrey) De Hoog & Scheffer; *Leptographium guttulatum* Jacobs & Wingfield; *Leptographium euphyes* Jacobs & Wingfield; *Leptographium alethinum* Jacobs, Wingfield, & Uzunovic; *Leptographium wingfieldii* Morelet; *Ophiostoma americanum* Jacobs, Wingfield, & Bergdahl; *Leptographium pyrinum* Davidson, *Leptographium hughesii* Jacobs, Wingfield, & Harrington, *Leptographium eucalyptophilum* Jacobs, Wingfield, & Roux; *Leptographium abicolens* Jacobs & Wingfield; *Ophiostoma franke-grosmanniae* (Davidson) De Hoog & Scheffer; *Ophiostoma penicillatum* (Grosmann) Siemaszko; and *Ophiostoma dryocoetidis* (Kendrick & Molnar) Harrington. *Leptographium costaricense* Weber, Spaaij, & Wingfield; *Leptographium pineti* Jacobs & Wingfield; and *Leptographium elegans* Wingfield, Crous, & Tzean clustered separately from this larger group. Both *L. elegans* and *L. pineti* were originally isolated from Asian countries. However, they occur on different host species.

The second group includes *Ophiostoma trinacriforme* (Parker) Harrington, *Leptographium brachiatum* (Kendrick) Wingfield, *Leptographium antibioticum* (Kendrick) Wingfield, and *Ophiostoma brevicolle* Davidson. Apart from *L. brachiatum* and *L. antibioticum*, which are morphologically similar, there is no obvious correlation between morphology and phylogeny within this group

The third group comprises *Leptographium reconditum* Jooste; *Ophiostoma laricis* Van der Westhuizen et al.; *Ophiostoma europhioides* (Wright & Cain) Solheim; and *Ophiostoma piceaperdum* (Rumbold) Arx; the three varieties of *Leptographium wageneri*; *Leptographium albopini* Wingfield, Harrington, & Crous; *Ophiostoma aureum* (Robinson-Jeffrey) Harrington; *Leptographium lundbergii* Lagerberg & Melin; *Leptographium pityophilum* Jacobs & Wingfield; *Leptographium procerum*; and *Leptographium terebrantis* Barras & Perry (Fig. 2). *Ophiostoma robustum* (Robinson-Jeffrey & Davidson) Harrington, *Ophiostoma grandifoliae* (Davidson) Harrington, and *Ophiostoma leptographioides* (Davidson) Arx clustered separately from this group.

Comparison of a subset of *Leptographium* species 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 outgroup. The shortest tree was 596 steps with a RI of 0.802 and CI of 0.542 (Fig. 3). The phylogram 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* species grouped closely together in the Ophiostomatales.

Morphological comparison

Analysis of the unweighted morphological characters using the heuristic search option in PAUP produced 1 tree. The shortest tree length was 1139 steps with a CI of 0.248 and RI of 0.752 (Fig. 4). No correlation could be found between trees produced based on molecular characters (Fig. 2) and those from the analysis of morphological characters (Fig. 4). 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 incongruent with those produced by the analyses of the unweighted characters (Fig. 4). Three morphologically important characters (conidiophore length, primary branch type, and conidium length to width ratio) were weighted preferentially. No correlation could be found between any of

Fig. 4. Analysis of the morphological characters using the heuristic search option in PAUP. (A) Analysis of the unweighted morphological characters produced one tree. The shortest tree length was 1139 steps with a CI of 0.248 and RI of 0.590. (B) Analysis of the morphological characters with conidiophore length as weighted character produced six trees. The shortest tree length was 1292 steps with a CI of 0.218 and RI of 0.577. (C) Analysis of the morphological characters with primary branch pattern as weighted character produced five trees. The shortest tree length was 1307 steps with a CI of 0.216 and RI of 0.509. (D) Analysis of the morphological characters with conidial dimensions as weighted character produced one tree. The shortest tree length was 1497 steps with a CI of 0.188 and RI of 0.418. The different fonts correspond to the three major groups derived from analysis of the molecular data and is indicated as such to simplify comparison with the analysis of the molecular data in Fig. 2.

the weighted trees based on morphology and those based on molecular characters. It is interesting, however, that *O. laricis*, *O. europhioides*, and *O. piceaperdum* clustered together as before. The same was true for the three varieties of *L. wageneri*.

The molecular and morphological data were also subjected to distance analysis using UPGMA. The resulting phenogram, as in the case of the parsimony analysis, was incongruent with the molecular data.

Discussion

DNA-based analysis of species in *Leptographium* has clarified the answers to 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 correlations emerged.

Based on analysis of the ribsomal DNA data, *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 et al. (1999*a*). 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 are similar in terms of disease, they occur on the same hosts and are associated with the same insects (Kendrick 1962; Barras and Perry 1971; Wingfield 1983; Highley and Tattar 1985; Alexander et al. 1988; Harrington 1988; Wingfield and 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 and Harrington 1992).

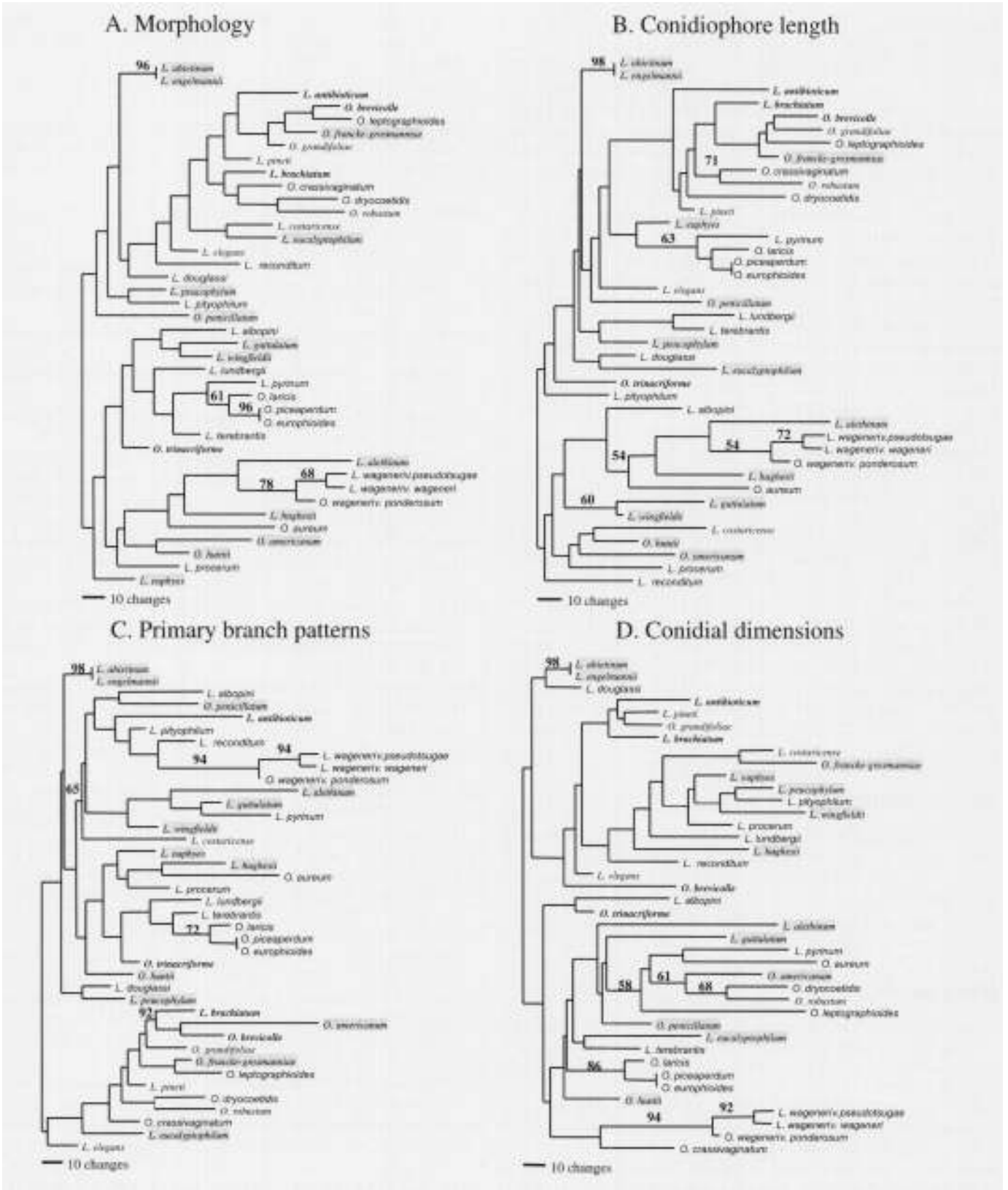
The outcome from the DNA sequence-based comparison in this study is similar to that of Zambino and Harrington (1992), which was based on isozyme analyses. Although these authors used a smaller number of species, a significant correlation can be seen between the results of the two studies. Species from the *L. serpens* and *L. lundbergii* cluster (Zambino and Harrington 1992) corresponded well to cluster III of the ribosomal DNA analysis in this study. *Leptographium albopini* (as *Leptographium* sp I), *L. aureum*, *L. terebrantis*, *L. lundbergii*, and *L. europhioides* formed part of the *L. lundbergii* group, 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 *L. engelmannii* with *L. abietinum*, as proposed by Zambino and Harrington (1992) and implemented by Jacobs et al. (1999*b*) 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 and Harrington 1992). This corresponds to clade II derived from the DNA analysis in the present study. The anamorph of *O. abiocarpum* was not described when the teleomorph of this fungus was described (Davidson 1966). However, Upadhyay (1981) reported a *Leptographium* anamorph for this species. We have not observed this morph in material available to us, but its close relationship with *Leptographium* in this study confirms that it fits appropriately with other *Leptographium* species. *Leptographium procerum* resides in the *L. serpens* group based on isozyme analysis (Zambino and Harrington 1992). However, from the DNA sequence data in this study, 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 and Harrington (1992).

Based on partial sequence of the ITS1 and ITS2, as well as the 5.8S gene, Jacobs et al. (2001) 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 tendency 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 Jacobs et al. (2001). The only discrepancy between the two studies was with the placement of *L. guttulatum*. Jacobs et al. (2001) 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 multistate code. Analyses of the un-



weighted characters produced a dendogram including two clades. Each clade included a group of species from the three main clusters emerging from the sequence analysis. The only groups in the resulting trees that were similar to those emerging from the molecular comparison were 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. Taxonomically important morpho-

logical characters, therefore, cannot be used to infer phylogeny in this genus, 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* species that are not known to be associated with a teleomorph, and those that have an *Ophiostoma* teleomorph. 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* species within the Ophiostomatales, together with *Ophiostoma* species. *Leptographium* species 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* species 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* species. 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 the future it will be desirable to extend this initial data base. We also hope that similar data sets for other genes will emerge and provide tools for the identification of *Leptographium* species.

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References

Alexander, S.A., Horner, W.E., and Lewis, K.J. 1988. *Leptographium procerum* as a pathogen of pines. *In Leptographium* root diseases on conifers. American Phytopathological Society, St. Paul, Minn. pp. 97–112.

Barras, S.J., and Perry, T. 1971. *Leptographium terebrantis* sp. nov. associated with *Dendroctonus terebrantis* in loblolly pine. Mycopatol. Mycol. Appl. **43**: 1–10.

Berbee, M.L., and Taylor, J.W. 1992. Convergence in ascospore discharge mechanism among pyrenomycete fungi based on 18s ribosomal RNA gene sequences. Mol. Phylogenet. Evol. **1**: 59–71.

Chen, W., Shrearer, C.A., and Crane, J.L. 1999. Phylogeny of *Ophioceras* spp. based on morphological and molecular data. Mycologia, **91**: 84–94.

Cobb, F.W., Jr. 1988. *Leptographium wageneri*, cause of black-stain root disease: a review of its discovery, occurrence and biology with emphasis on pinyon and ponderosa pine. *In Leptographium* root diseases on conifers. American Phytopathological Society, St Paul, Minn. pp. 41–62.

Davidson, R.W. 1966. New species of *Ceratocystis* from conifers. Mycopatol. Mycol. Appl. **28**: 273–286.

Dupont, J., Laloui, W., and Roquebert, M.F. 1998. Partial ribosomal DNA sequences show an important divergence between *Phaeoacremonium* species isolated from *Vitis vinifera*. Mycol. Res. **102**: 631–637.

Guadet, J., Julien, J, Lafay, J.F., and Brygoo, Y. 1989. Phylogeny of some *Fusarium* species, as determined by large-subunit rRNA sequence comparison. Mol. Biol. Evol. **6**: 227–242.

Glenn, A.E., Bacon, C.W. Price, R., and Hanlin, R.T. 1996. Molecular phylogeny of *Acremonium* and its taxonomic implications. Mycologia, **88**: 369–383.

Guého, E., Kurtzman, C.P., and Peterson, S.W. 1989. Evolutionary affinities of Heterbasidiomycetous yeasts estimated from 18S and 25S ribosomal RNA sequence divergence. Syst. Appl. Microbiol. **12**: 230–236.

Harrington, T.C. 1981. Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. Mycologia, **73**: 1123–1129.

Harrington, T. C. 1988. *Leptographium* species, their distributions, hosts and insect vectors. *In Leptographium* root diseases on conifers. American Phytopathological Society, St. Paul, Minnesota. pp. 1–39.

Harrington, T.C., and Cobb, F.W., Jr. 1983. Pathogenicity of *Leptographium* and *Verticicladiella* spp. isolated from roots of Western North American conifers. Phytopathology, **73**: 596–599.

Hausner, G., Reid, J., and Klassen, G.R. 1992. Do galeate-ascospore members of the Cephaloascaceaea, Endomycetaceae and Ophiostomataceae share a common phylogeny? Mycologia, **84**: 870–881.

Hausner, G., Reid, J., and Klassen, G.R. 1993*a*. On the subdivision of *Ceratocystis* s.l., based on partial ribosomal DNA sequences. Can. J. Bot., **71**: 52–63.

Hausner, G., Reid, J., and Klassen, G.R. 1993*b*. *Ceratocystiopsis*: a reappraisal based on molecular criteria. Mycol. Res. **97**: 625–633.

Hibbett, D., and Vilgalys, R. 1993. Phylogenetic relationships of *Lentinus* (Basidiomycotina) inferred from molecular and morphological characters. Syst. Bot. **18**: 409–433.

Highley, L., and Tattar, T.A. 1985. *Leptographium terebrantis* and black turpentine beetles associated with blue stain and mortality of black and Scots pines on Cape Cod, Massachusetts. Plant Dis. **69**: 528–530.

Horner, W.E., Alexander, S.A., and Julian, M.M. 1986. Qualitative determination of cellulose in the cell walls of *Verticicladiella procera*. Mycologia, **78**: 300–303.

Jacobs, K.A., and Rehner, S.A. 1998. Comparison of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. Mycologia, **90**: 601–610.

Jacobs, K., Wingfield, M.J., and Bergdahl, D. 1997. A new species of *Ophiostoma* from North America, similar to *Ophiostoma penicillatum*. Can. J. Bot. **75**: 1315–1322.

Jacobs, K., Wingfield, M.J, Wingfield, B.D., and Yamaoka, Y. 1998. Comparison of *Ophiostoma huntii* and *O. europhioides* and description of *O. aenigmaticum* sp. nov. Mycol. Res. **102**: 289–294.

Jacobs, K., Wingfield, M.J., and Crous, P.W. 1999*a*. *Ophiostoma europhioides* and *Ceratocystis pseudoeurophioides*, synonyms of *O. piceaperdum*. Mycol. Res. **104**: 238–243.

Jacobs, K., Wingfield, M.J., Harrington, T.C., and P.W. Crous. 1999*b*. *Leptographium engelmannii*, a synonym of *Leptographium abietinum*, and description of *Leptographium hughesii* sp. nov. Can. J. Bot. **76**: 1660–1667.

Jacobs, K., Wingfield, M.J., Coetsee, C., Kirisits, T., and Wingfield, B.D. 2001. *Leptographium guttulatum* sp.nov., a new species from spruce and pine in Europe. Mycologia, **93**: 380–388.

Jacobs, K., and Wingfield, M.J. 2001. *Leptographium* species: tree pathogens, insect associates and agents of blue-stain. American Phytopathological Society Press, St. Paul, Minn. pp. 1–207.

Kellogg, E.A., Appels, R., and Mason-Gamer, R.J. 1996. When genes tell different stories: the diploid genera of Triticeae. Syst. Bot. **21**: 321–347.

Kendrick, W.B. 1962. The *Leptographium* complex. *Verticicladiella* S. Hughes. Can. J. Bot. **40**: 771–797.

Kurtzman, C.P. 1993. Systematics of the ascomyceteous yeasts assessed from ribosomal RNA sequence divergence. Antonie Leeuwenhoek, **63**: 165–174.

Kurtzman, C.P. and Robnett, C.J. 1991. Phylogenetic relationships among species of *Saccharomyces*, *Schizosaccharomyces*, *Debaromyces* and *Schwanniomyces* determined from partial ribosomal RNA sequences. Yeast, **7**: 61–72.

Lorenzini, G., and Gambogi, P. 1976. Decline of *Pinus pinea* associated with the presence of *Verticicladiella* sp. (preliminary note) [In Italian.] Inf. Fitopatol. **5**: 5–8.

Marais, G.J. 1996. Fungi associated with infructescences of the *Protea* species with special reference to the Ophiostomatales. Ph.D. thesis, Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein, South Africa.

Myburg, H., Wingfield, B.D., and Wingfield, M.J. 1999. Phylogeny of *Cryphonectria cubensis* and allied species inferred from DNA analysis. Mycologia, **91**: 243–350.

O'Donnell, K. 2000. Molecular phylogeny of the *Nectria haematococca – Fusarium solani* species complex. Mycologia, **92**: 919–938.

O'Donnell, K., Cigelnik, E., and Nirenberg, H.I. 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia, **90**: 465–493.

Okada, G., Takematsu, A., and Takamura, Y. 1997. Phylogenetic relationships of the hyphomycete genera *Chaetopsina* and *Kionochaeta* based on 18S rDNA sequences. Mycoscience, **38**: 409–420.

Patterson, C., Williams, D.M., and Humphries, C.J. 1993. Congruence between molecular and morphological phylogenies. Annu. Rev. Ecol. Syst. **24**: 153–188.

Peterson, S.W., and Kurtzman, C.P. 1991. Ribosomal RNA sequence divergence among sibling species of yeasts. Syst. Appl. Microbiol. **14**: 124–129.

Raeder, U., and Broda, P. 1985. Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. **1**: 17–20.

Saiki, R.J., Gelfand, D.A., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science (Washington, D.C.), **239**: 487–491.

Solheim, H. 1986. Species of Ophiostomataceae isolated from *Picea abies* infested by the bark beetle *Ips typographus*. Nord. J. Bot. **6**: 199–207.

Solheim, H. 1993. Fungi associated with the spruce bark beetle *Ips typographus* in an endemic area in Norway. Scand. J. For. Res. **8**: 118–122.

Strydom, R.C., Wingfield, B.D., and Wingfield, M.J. 1997. Ribosomal DNA sequence comparison of *Leptographium lundbergii* and *L. truncatum* and neotypification of *L. lundbergii*. Syst. Appl. Microbiol. **20**: 295–300.

Swofford, D.L. 1999. PAUP* (version 4.0) Phylogenetic Analysis Using Parsimony, version 3.1.1 edition. Illinois Natural History Survey, Champaign, Ill.

Upadhyay, H.P. 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press, Athens, Ga.

Van der Westhuizen, K., Wingfield, M.J., Yamaoka, Y., Kemp, G.H.J., and Crous, P.W. 1995. A new species of *Ophiostoma* with a *Leptographium* anamorph from Larch in Japan. Mycol. Res. **99**: 1334–1338.

Vilgalys, R., and Sun, B.L. 1994. Ancient and recent patterns of geographic speciation in the oyster mushroom *Pleurotus* revealed by phylogenetic analysis if ribosomal DNA sequences. Proc. Natl. Acad. Sci. U.S.A. **91**: 4599–4603.

Viljoen, C.D. 1996. A taxonomic study of *Ceratocystis* s.l. with special reference to species associated with *Protea* infructescences in Southern Africa. Ph.D. thesis, University of the Orange Free State, Bloemfontein, South Africa.

Wagener, W.W., and Mielke, J.L. 1961. A staining fungus root disease of ponderosa, Jeffrey and pinyon pines. Plant Dis. Rep. **45**: 831–835.

Ward, E., and Adams, M.J. 1998. Analysis of ribosomal DNA sequences of *Polymyxa* species and related fungi and the development of genus- and species- specific PCR primers. Mycol. Res. **102**: 965–974.

Webber, J.F., Jacobs, K., and Wingfield, M.J. 1999. A re-examination of the vascular wilt pathogen of takamaka (*Calophyllum inophylllum*). Mycol. Res. **103**: 1588–1592.

White, T.J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* PCR protocols: a guide to methods and applications. *Edited by* A.M. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White. Academic Press, San Diego, Calif.

Wingfield, M.J. 1983. Association of *Verticicladiella procera* and *Leptographium terebrantis* with insects in the Lake states. Can. J. For. Res. **13**: 1238–1245.

Wingfield, M.J. 1993. *Leptographium* species as anamorphs of *Ophiostoma*: progress in establishing acceptable generic and species concepts. *In Ceratocystis* and *Ophiostoma*. Taxonomy, ecology and pathogenicity. *Edited by* M.J. Wingfield. K.A. Seifert, and J.F. Webber. American Phytopathological Press, St. Paul, Minn. pp. 43–51.

Wingfield, M.J., and Gibbs, J.N. 1991. *Leptographium* and *Graphium* species associated with pine-infesting bark beetles in England. Mycol. Res. **95**: 1257–1260.

Wingfield, M.J., and Knox-Davies, P.S. 1980. Root-disease, associated with *Verticicladiella alacris*, of pines in South Africa. Plant Dis. **64**: 569–571.

Wingfield, M.J., Capretti, P., and Mackenzie, M. 1988. *Leptographium* spp. as root pathogens on conifers. An international perspective. *In Leptographium* root diseases on conifers. American Phytopathological Society, St. Paul, Minn. pp. 113–128.

Wingfield, B.D., Grant, W.S., Wolfaardt, J.F., and Wingfield., M.J. 1994*a*. Ribosomal RNA sequence phylogeny is not congruent with ascospore morphology among species in *Ceratocystis* sensu stricto. Mol. Biol. Evol. **11**: 376–383.

Wingfield, M.J., Crous, P.W., and Tzean, S.S. 1994*b*. *Leptographium elegans*: a new species from Taiwan. Mycol. Res. **98**: 781–785.

Wingfield, M.J., De Beer, C., Visser, C., and Wingfield, B.D. 1996. A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. Syst. Appl. Microbiol. **19**: 191–202.

Wingfield, B.D., Viljoen C.D., and Wingfield, M.J. 1999. Phylogenetic relationships of ophiostomatoid fungi associated with *Protea* infructescences in South Africa. Mycol. Res. **103**: 1616–1620.

Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J., and Wolfaardt, M. 1998. Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. Mycologia, **90**: 96–101.

Yamada, Y., and Kawasaki, H. 1989. The molecular phylogeny of the Q_8-equipped basidiomycetous yeast genera *Mrakia* Yamada et Komagata and *Cystofilobasidium* Oberwinkler et Bandoni based on the partial sequences of 18S and 26S ribosomal ribonucleic acids. J. Gen. Appl. Microbiol. **35**: 173–183.

Yamada, Y., Kawasaki, H., Nakase, T., and Banno, I. 1989. The phylogenetic relationship of the conidium forming anamorphic yeast genera *Sterigtomyces*, *Kurtzmanomyces*, *Tsuchiyaea*, and *Fellomyces*, and the teleomorphic yeast genus *Sterigtomatosporidium* on the basis of the partial sequences of 18S and 26S ribosomal ribonucleic acids. Agric. Biol. Sci. **53**: 2993–3001.

Zambino, P.J. and Harrington, T.C. 1992. Correspondence of isozyme characterisation with morphology in the asexual genus *Leptographium* and taxonomic implications. Mycologia, **84**: 12–25.