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Phylogenetic relationships among *Phialocephala* species and other ascomycetes

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Abstract: *Phialocephala* was established for species in the *Leptographium* complex that produce conidia from phialides at the apices of dark mononematous conidiophores. Some species previously included in *Phialocephala* were re-allocated to *Sporendocladia* because they resembled *Thielaviopsis* in having ring-wall-building conidial development and conidia with two attachment points that emerge in false chains. Despite this significant realignment of the genus, a great deal of morphological heterogeneity remains in *Phialocephala*. The objective of this study was to consider the heterogeneity among *Phialocephala* spp. based on comparisons of sequence data derived from the large and small subunits (LSU and SSU) of the rRNA operon of species in *Phialocephala*. *Phialocephala dimorphospora*, the type species of the genus, and *P. fortinii* grouped with genera of the Helotiales in phylogenetic trees generated based on the LSU and SSU datasets. *Phialocephala xalapensis* and *P. fusca* clearly are unrelated to *Phialocephala sensu stricto* and should represent a new genus in the Ophiostomatales. *Phialocephala compacta* resides with representatives of the Hypocreales, and we believe that it represents a distinct genus. *Phialocephala scopiformis* and *P. repens* are not closely related to the other *Phialocephala* species and group within the Dothideales. The morphological heterogeneity among species of *Phialocephala* clearly is reflected by phylogenetic analysis of sequence data from two conserved rRNA gene regions. Appropriate genera now need to be found to accommodate these fungi.

Key words: *Leptographium*, morphology, *Phialophora*, phylogeny

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INTRODUCTION

Phialocephala Kendrick was established to accommodate species in the *Leptographium* Lundberg & Melin complex, which produce conidia in phialides (Kendrick 1961). This distinguishes them from *Leptographium* spp. that are characterized by percurrent or sympodial proliferation of the conidiogenous cells (Jacobs and Wingfield 2001). *Phialocephala* spp. are further characterized by having dark mononematous conidiophores that branch penicillately at their apices (Crane 1971) and thus resemble *Phialophora* Medlar (Gams 2000). Hyaline ameroconidia accumulate in slimy masses around the sporogenous heads (Kendrick 1961, 1963). In addition, some species produce solitary phialides that are formed directly on the mycelium (Onofri and Zucconi 1984). The so-called ''stalked spore drop'' structure, as described by Ingold (1961), suggests an adaptation to insect dispersal, although insect associations are not known for most species of *Phialocephala* (Jacobs and Wingfield 2001).

Phialocephala spp. occupies a diverse range of ecological niches (Wang and Wilcox 1985, Kowalski and Kehr 1995). *Phialocephala dimorphospora* W.B. Kendrick, *P. fortinii* C.J.K. Wang & H.E. Wilcox, *P. compacta* T. Kowalski & R.D. Kehr and *P. scopiformis* T. Kowalski & R.D. Kehr are readily isolated from plants growing in cool or cold environments, such as those encountered in alpine, subalpine and boreal regions (Wang and Wilcox 1985, Hambleton and Currah 1997, Stoyke and Currah 1990). *Phialocephala trigonospora* R. Kirschner & F. Oberwinkler was isolated from bark beetle tunnels in *Pinus sylvestris* L. and *Picea abies* L. Karst., while *P. scopiformis* and *P. compacta* are endophytes of *Pinus* and *Picea* spp. (Kowalski and Kehr 1995, Kirschner and Oberwinkler 1998). Most species are not associated with disease, but *P. virens* A.L. Siegfried & K.A. Seifert was isolated from root rot on *Tsuga* and *Picea* spp. (Siegfried et al 1992). *Phialocephala fortinii* also has been reported as a weak pathogen of container-grown conifers (Wil-

cox and Wang 1987). The latter fungus is well known as *Mycelium radicis atrovirens* Melin, which colonizes tree roots (Wang and Wilcox 1985).

No teleomorph associations have been determined for species of *Phialocephala*, although a connection to the Leotiales has been proposed (Currah et al 1993). This was based on the morphology of apothecium-like structures with cells resembling immature asci, produced in some cultures (Currah et al 1993). Previously, the anamorph of *Ophiostoma francke grosmanniae* R.W. Davidson also was suggested to represent a *Phialocephala* species. However, the presence of closely packed annellations, observed in an ultrastructural study, showed that the anamorph of this fungus rather should reside in *Leptographium* (Mouton et al 1992), an assignment that was confirmed in a recent phylogenetic study of *Leptographium* spp. based on DNA sequence data (Jacobs et al 2001).

The morphologically heterogeneous nature of *Phialocephala* was emphasised when the species with inconspicuous collarettes and ring-wall-building conidial development were moved to *Sporendocladia* G. Arnaud ex Nag Raj & W.B. Kendr. (Wingfield et al 1987). However, based on morphological and physiological variability, the remaining *Phialocephala* spp. still represents a heterogeneous group. The variable morphological characteristics include a wide diversity of conidial forms and variously structured collarettes at the apices of conidiogenous cells. Furthermore, the variable presence of rhizoids at the base of conidiophores and sterile outgrowths on the stipes suggest that many of these fungi phylogenetically are unrelated.

Phialocephala fusca W.A. Kendrick is the only *Phialocephala* sp. that forms rhizoids at the base of conidiophore stipes (Kendrick 1963). Likewise, *P. canadensis* W.A. Kendrick and *P. fluminis* C.A. Shearer, J.L. Crane & M.A. Miller are unique in that they have sterile outgrowths on stipes (Kendrick 1963, Shearer et al 1976). Collarette morphology in *Phialocephala* spp. varies from being broadly flared in *P. fusca* to deeply set in *P. dimorphospora* and inconspicuous in *P. humicola* Jong & E. Davis (= *P. gabalongii* Sivasith.) (Kendrick 1961, 1963, Jong and Davis 1972). Conidial shapes in *Phialocephala* spp. range from ellipsoidal to globose and subglobose. Some species have two distinct forms of conidia. The first-formed conidium that develops fully inside a very long collarette is larger than the second and subsequent conidia. This dimorphism is present in *P. dimorphospora*, *P. fortinii*, *P. compacta* and *P. scopiformis*, while one species, *P. trigonospora*, has uniquely triangular spores (Kirschner and Oberwinkler 1998).

Phialocephala spp. varies in tolerance to the antibiotic cycloheximide. This tolerance to cycloheximide might indicate connections to the Ophiostomatales (Harrington 1988, Jacobs and Wingfield 2001). Species such as *P. dimorphospora* displays 84%, *P. fortinii* 55% and *P. humicola* 60% reduction in growth in the presence of 0.5 g/L of cycloheximide. *Phialocephala fusca*, *P. repens* and *P. xalapensis* will not grow in the presence of the antibiotic (Jacobs 2000).

Very limited molecular data are available from which to infer phylogenetic relationships for *Phialocephala* spp. The only species that have been considered at this level are *P. fortinii* and *P. dimorphospora* (Rogers et al 1999). Based on ITS sequence comparisons, these two species appear to be closely related. This relationship also is supported strongly by morphological and ecological characteristics.

The aim of this study was to consider phylogenetic relationships between *Phialocephala* spp. for which cultures are available. In addition, we evaluated the placement of *Phialocephala* spp. within orders of the Ascomycota. These objectives were achieved by means of analyzing partial sequences of the SSU and LSU genes of the ribosomal RNA operon.

MATERIALS AND METHODS

Fungal isolates.—Isolates were obtained from a wide variety of sources (TABLE I). All isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction.—Isolates were grown in liquid malt extract (ME) (2% w/v, NT Merck) at 25 C in the dark for 14 d, harvested by centrifugation (13 000 × *g*) and lyophilized. DNA was isolated using a modification of the DNA extraction procedure of Raeder and Broda (1985). Mycelium was ground to a fine powder in liquid nitrogen, to which 1 mL extraction buffer (200 mM Tris-HCl, pH 8; 25 mM EDTA, pH 8; 150 mM NaCl; and 0.5% SDS) was added. This was followed by further homogenization and incubation (1 h, 60 C). Cell debris was precipitated by centrifugation (ca 15 700 × *g*, 30 min). A series of phenol:chloroform (0.5 v/v) extractions were performed until the interface was clean. Nucleic acids were precipitated in cold 100% ethanol (2:1 v/v) and incubated at −20 C for 24 h. The mixture subsequently was centrifuged (15 700 × *g*, 30 min) and washed in 70% ethanol. The pellet was resuspended in 300 μL sterile water.

PCR.—Extracted DNA was used as template in a PCR reaction to amplify regions of the nuclear LSU and the SSU genes of the ribosomal RNA (rRNA) operon. The SSU gene was amplified using primer sets 2F (5′-ATCTGGTTGATCC TGCCAGTAG-3′) and 1794R (5′-GATCCTTCCGCAGG TTCACC-3′) (Okada et al 1997). The ITS 2 region and a portion of the LSU gene were amplified using the primer set CS3 (5′-CGAATCTTTGAACGCACATTG-3′) (Visser et al 1995) and LR3 (5′-CCGTGTTTCAAGACGGG-3′) (White et al 1990). The PCR reaction mixture included $MgCl_2$ (2.5

TABLE I. List of fungi for which sequence data were generated in this study

Culture number[a]	Alternative designation[b]	Name	Origin	Collector	GenBank accession number	
					Small subunit	Large subunit
CMW 4946	CBS 507.94 ATCC 96754	*P. compacta* Kowalski & Kehr	Braunschweig, Germany	T. Kowalski	AF326072	AF326083
CMW508 (A)	ATCC 24087	*P. dimorphospora* W.B. Kendr.	Maryland, U.S.A.	C.A. Shearer	AF26070	AF326081
CMW168 (B)		*P. dimorphospora* W.B. Kendr.	Maryland, U.S.A.	C.A. Shearer	AF326069	AF326080
CMW5590	CBS 443.86 ATCC 60614	*P. fortinii* W.B. Kendr.	Suonenjoki, Finland	C.J.K. Wang	AF26071	AF326082
CMW 172	CBS 301.85 ATCC 62326	*P. fusca* W.B. Kendr.	Ottawa, Canada	W.B. Kendrick	AF326067	AF326078
CMW5339	MUCL 1849	*P. repens* (Cooke & Ellis) W.B. Kendr.	Regensberg, Germany	T. Kowalski	AF326073	AF326084
CMW 4947	CBS 468.94 ATCC 96754	*P. scopiformis* Kowalski & Kehr	Regensberg, Germany	T. Kowalski	AF32674	AF326085
CMW 5594	CBS 218.86	*P. xalapensis* Persiani & Maggi	India	V. Rao	AF326068	AF326079
CMW 30		*L. lundbergii* Lagerb. & Melin	New Zealand	M. Dick	AF26066	AF326077
CMW 1593	IFO 8852	*S. bactrospora* (W.B. Kendr.) M.J. Wingf.	Japan	M. Ichinoe	AF26065	AF326076

[a] CMW refers to the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[b] IFO refers to the culture collection of the Institute for Fermentation, Osaka, Japan. CBS refers to the culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. ATCC refers to the culture collection of the American Type Culture Collection, U.S.A. MUCL refers to the culture collection of the Belgian Co-ordinated Collections of Micro-organisms, Louvais-la-Neuve, Belgium.

TABLE II. Species included in phylogenetic analyses for which sequence data were derived from GenBank

Species	Order	GenBank accession number (LSU)	GenBank accession number (SSU)
Ascolacicola austriaca Réblová, Winka & Jakl.	Trichotheliales	AF261067	AF242263
Beauveria bassiana (Bals.-Criv.) Vuill.	Hypocreales	AF049164	AB079126
Botryosphaeria ribis Grossenb. & Duggar	Dothideales		U42477
Ceratocystis fimbriata (Ellis & Halst) Sacc.	Microascales	U17401	U32418
Cercophora septentrionalis N. Lundq.	Sordariales	U47823	U32400
Chaetomium globosum Kunze	Sordariales	U47825	U20379
Chaetopsina fulva Rambelli	Hypocreales		AB003786
Chromocleista malachitea Yaguchi & Udagawa	Eurotiales	AB000621	D88323
Colletotrichum trifolii Bain.	Phyllachorales	AJ301942	AJ301942
Cordyceps tuberculata (Lebert) Maire	Hypocreales	AF327384	AF327401
Diaporthe phaseolorum (Cooke & Ellis) Sacc.	Diaporthales	U47830	L36985
Dothidea ribesia Pers.	Dothideales	AY016360	AY016343
Evernia prunastri (L.) Ach.	Lecanorales	AF107562	AF117987
Fonsecaea pedrosoi (Brumpt) Negroni		L36997	AF050276
Glomerella cingulata (Stoneman) Spauld. & H. Schrenke	Phyllachorales	AF222490	AF222531
Hamigera avellanea Stolk & Samson	Eurotiales	D14406	AB000620
Haptocillium balanoides (Drechsler) Zare & W. Gams	Hypocreales	AF339541	AF339590
Hypocrea schweinitzii (Fr.) Sacc.	Hypocreales	L36986	U47833
Hypomyces chrysospermus Tul. & C. Tul.	Hypocreales	AF160233	M89993
Leotia viscose Fr.	Helotiales	AF113737	AF113715
Magnaporthe grisea (T.T. Hebert) M.E. Barr		AB026819	AF056626
Microascus cirrosus Curzi	Microascales	AF275540	AF275525
Mycosphaerella mycopappi A. Funk & Dorworth	Dothideales		U43463
Ophiostoma piliferum (Fr.) Syd. & P. Syd.	Ophiostomatales	U47837	U20377

mM), Expand HF buffer without $MgCl_2$, dNTPs (0.2 mM each), primers (0.025 mM), template DNA (25 ng) and Expand™ High Fidelity PCR System (1.75 U) (Roche Pharmaceuticals, Germany). The PCR reaction conditions for the amplification of the LSU were an initial denaturation at 94 C for 2 min, annealing at 48 C for 1 min, ramping at 5 C/s to 72 C for 2 min. This was repeated for 40 cycles, and a final elongation step was included at 72 C for 8 min. The SSU was amplified following the same PCR reaction conditions but only for 25 cycles. The resulting PCR amplicons were purified with a QIAquick PCR Purification kit (QIAGEN, Germany), according to specifications of the manufacturer.

DNA sequencing.—DNA sequences were determined with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Applied Biosystems, UK), using primer sets CS3, LR3 and 2F, 1794R. Two internal primers 404X (5′-CCCTTTCAACAATTTCAC-3′) (Witthuhn et al 1999) and 1332R (5′- AAGGTCTCG TTCGTTATCG-3′) (Okada et al 1997) were included for the large and small subunit, respectively. Sequences generated in this study have been deposited in GenBank (TABLE I).

Sequence analysis.—Alignments of the LSU and SSU datasets were obtained by means of the Clustal X (Thompson et al 1994) program, and the inserted gaps were treated as "new state". Ambiguously aligned regions and parsimony-uninformative characters were excluded from the datasets. The remaining characters were reweighted according to the mean consistency index (CI). Phylogenetic analysis was based on parsimony using PAUP 4.0* (Phylogenetic Analysis Using Parsimony* and Other Methods version 4 (Swofford 1998). Heuristic searches were conducted with random addition of sequences (100 replicates), tree bisection-reconnection (TBR) branch swapping and MULPAR effective and MaxTrees set to auto-increase. Phylogenetic signal in the datasets ($g1$) was assessed by evaluating tree length distributions over 100 randomly generated trees (Hillis and Huelsenbeck 1992). The CI and retention indexes (RI) were determined for all datasets. Phylogenetic trees were rooted with *Xylaria curta* as a monophyletic sister outgroup to the rest of the taxa. Bootstrap analyses were performed to determine confidence in branching points (1000 replicates) for the most-parsimonious (MP) trees generated from the SSU and LSU data. The combinability of the SSU and LSU datasets were tested using the partition-homogeneity test and the Templeton Nonparametric Wilcoxon Signed Ranked test in PAUP 4.0 (Farris et al 1994, Kellogg 1996). The datasets were submitted to Treebase (SN904–3213).

RESULTS

Statistical analysis to determine combinability.—The partition-homogeneity test of the combined SSU and

LSU datasets showed sufficient probability for rejecting the null hypothesis ($P < 0.05$). This indicates incongruence of the two datasets and results thus are presented separately in the following sections. This lack of congruence also was indicated by the Templeton Nonparametric Wilcoxon Signed Ranked (WSR) test. The LSU and SSU data thus are represented separately.

SSU sequence data.—Parsimony analysis of the SSU sequence data was done to determine the phylogenetic placement of *Phialocephala* species in relation to representatives of different orders in the Ascomycetes. Alignment by inserting gaps resulted in a total of 423 characters used in the comparison of the different species. The inserted gaps were treated as ''new state'' and all parsimony-uninformative and ambiguous characters were excluded. The remaining characters were reweighted according to the mean CI value. A total of 100 parsimony-informative characters were obtained. Heuristic searches on the dataset generated 100 MP trees and a single tree is presented in FIG. 1.

Phialocephala dimorphospora and *P. fortinii* grouped together and apart from the other *Phialocephala* species. They grouped basal to the clade representing the Lecanorales, although the association is not supported by bootstrap values. An isolate of *Sporendocladia bactrospora* was placed in the Microascales clade together with *Ceratocystis fimbriata* (Ellis & Halst.) Sacc. *Phialocephala compacta* grouped basal to representatives of the Hypocreales, while *Phialocephala repens* and *P. scopiformis* grouped separately from all the other *Phialocephala* species, showing similarities to representatives of the Dothideales. The relationship between *P. repens* and *P. scopiformis* was well supported by the bootstrap values obtained. *Phialocephala xalapensis* and *P. fusca* formed part of the Ophiostomatales cluster. The relationship between these two *Phialocepala* species and representatives of the Ophiostomatales is supported by a relative low (79%) bootstrap value.

LSU sequence data.—Alignment of the LSU gene sequences was achieved by inserting gaps. These gaps were treated as ''new state'', and all ambiguous and parsimony-uninformative characters were excluded. The remaining characters were reweighted according to the mean CI value. A total of 100 parsimony-informative characters were used in the comparison of the different species. Heuristic searches on the dataset generated a single MP tree. The tree obtained is presented in FIG. 2.

Analysis of the LSU sequence data generally reflected relationships determined based on SSU data. *Phialocephala dimorphospora* and *P. fortinii* grouped distantly with the Lecanorales. *Sporendocladia bactrospora* remained well placed in the Microascales. The placement of *P. compacta*, *P. scopiformis* and *P. repens* could not be established using this dataset, although *P. compacta* grouped basal to the Hypocreales. *Phialocephala xalapensis* and *P. fusca* formed an independent clade related to the Sordariales. This relationship is not supported by bootstrap values.

DISCUSSION

Results of this study confirm views that, based on morphology and ecology, species of *Phialocephala* are phylogenetically unrelated. Analyses of sequence data thus have shown that species considered in this study most probably represent taxa in the Lecanorales, Ophiostomatales, Hypocreales and Dothideales. Although this was not an objective of this study, it became evident that various species currently residing in *Phialocephala* clearly require new genera.

Phialocephala dimorphospora is the type species of the genus. The fungus has characteristic deeply set conidiogenous loci with tubular collarettes (Kendrick 1961). This is very similar to species of *Cystodendron* Bubák, and the relatedness of *Phialocephala* and *Cystodendron* should be considered in future studies. *Cystodendron* is characterized by dark, densely penicillate and more or less sporodochial conidiophores. The phialides have pronounced tubular collarettes. *Phialocephala fortinii* has conidiophores and conidia similar to those of *P. dimorphospora*, but its sporulation is scanty and occurs only at low temperatures, and the two fungi share similar ecological niches. It was not surprising, therefore, that the two fungi are found to be phylogenetically related. Furthermore, our results support those of a previous study that has suggested that these fungi probably reside in the Leotiales (Rogers et al 1999, Currah et al 1993). The low bootstrap values obtained for the relatedness of the two *P. dimorphospora* isolates suggest that there is variability in isolates of this fungus and this matter deserves further study. The relationship, however, is supported strongly by the more variable LSU dataset.

In many respects, *Phialocephala* spp. is morphologically similar to *Leptographium* species. Species in both genera have erect conidiophores with conidia produced in slimy masses at the apices of branched conidiogenous cells. In *Leptographium*, this morphological form is known to facilitate an association with insect vectors (Jacobs and Wingfield 2001). Thus it is not surprising that two *Phialocephala* species (*P. fusca* and *P. xalapensis*) included in this study were found to be related to *Leptographium* in the Ophiostomatales. However, both species lack tolerance to 0.5 g/L cycloheximide, which is unlike typical *Lepto-*

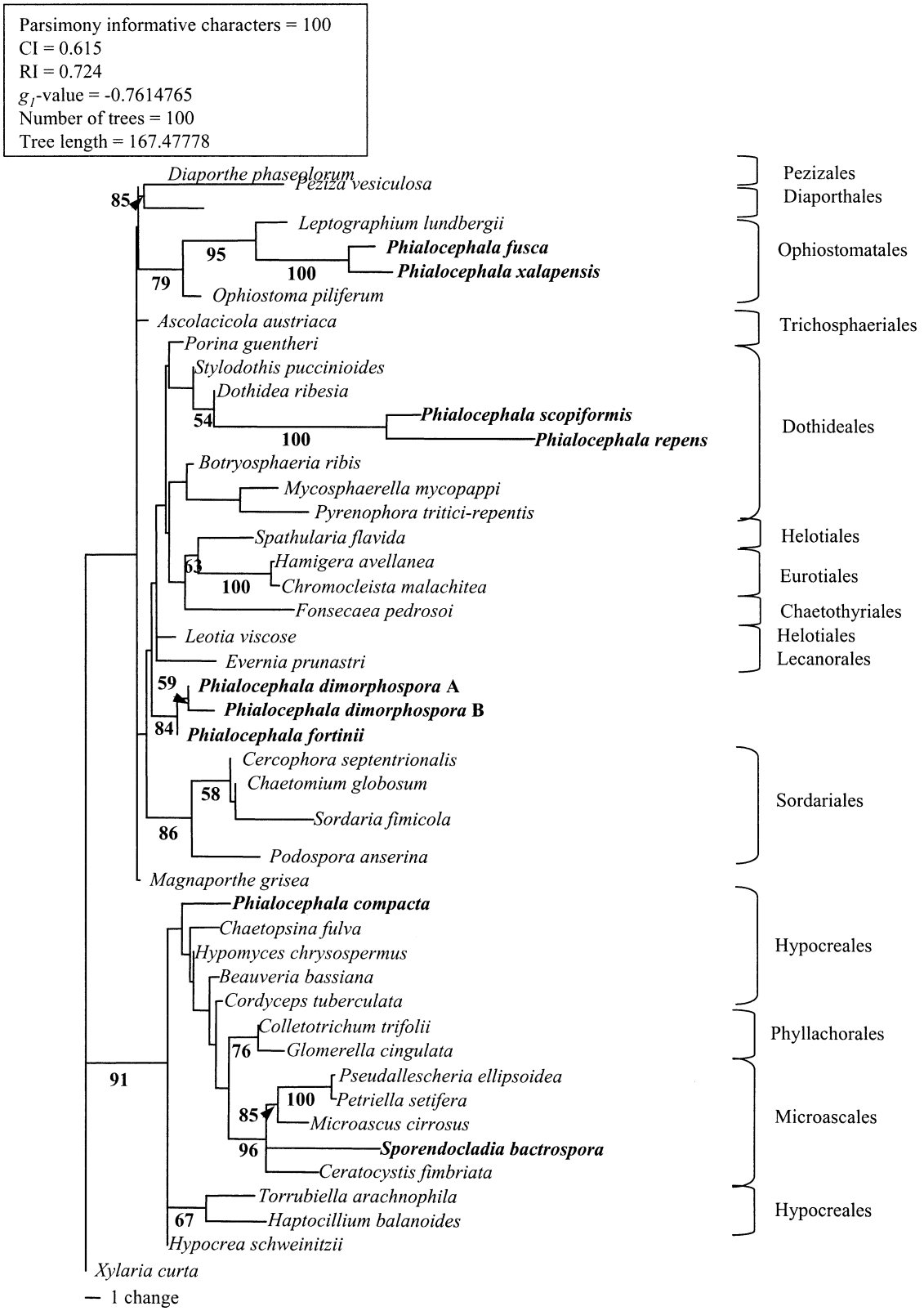

FIG. 1. Phylogenetic tree (tree No. 3) produced by PAUP* heuristic option of the SSU rDNA with *Xylaria curta* as outgroup. Bootstrap values above 50% (percentages of 1000 bootstrap replicates) are indicated below the branches of the tree in bold print.

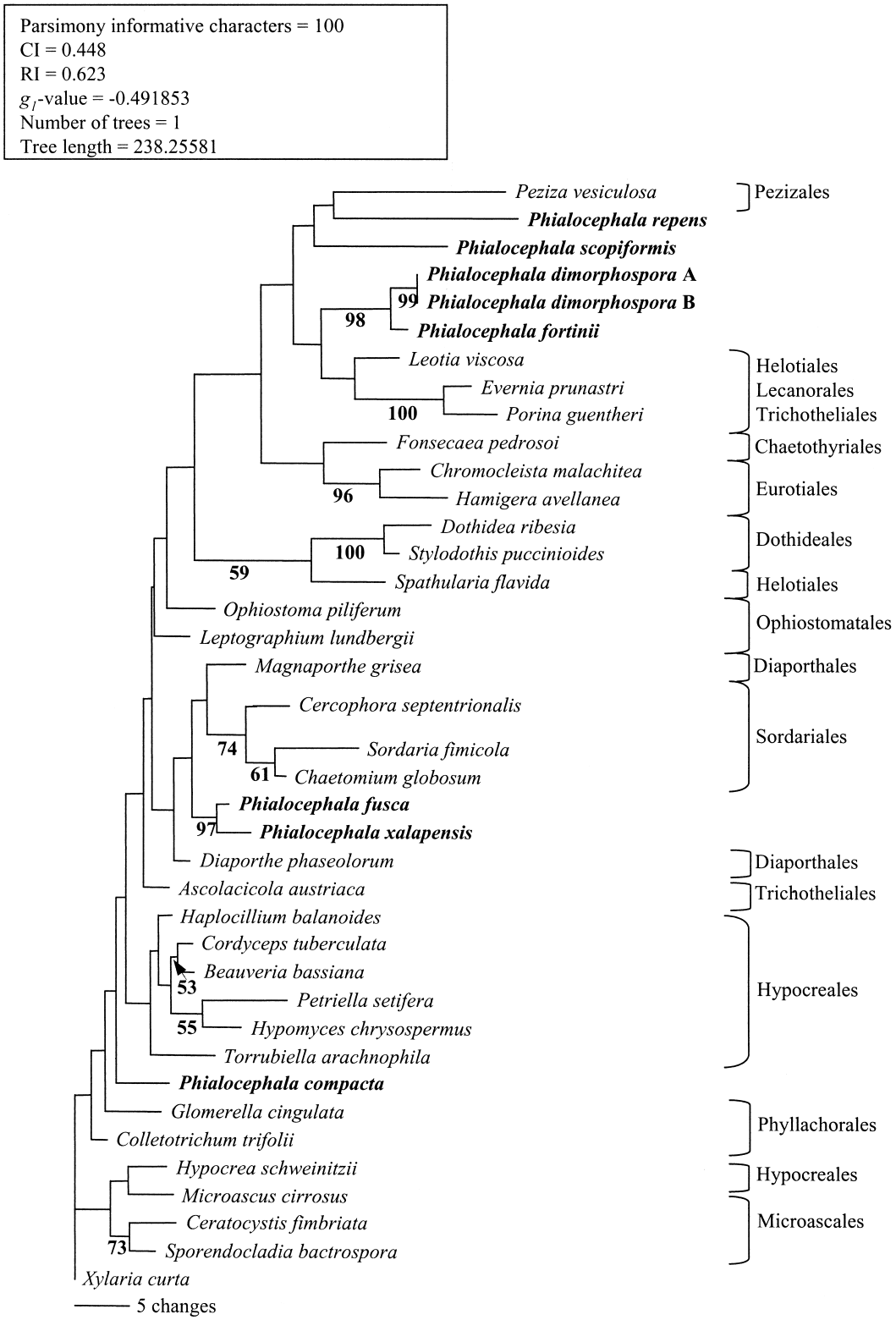

FIG. 2. Phylogenetic tree produced by PAUP* heuristic option of the LSU rDNA with *Xylaria curta* as outgroup. Bootstrap values above 50% (percentages of 1000 bootstrap replicates) are indicated below the branches of the tree in bold print.

graphium spp. (Jacobs and Wingfield 2001) and might represent a new genus associated with Ophiostomatoid fungi.

Loss of collarettes in a number of *Phialocephala* spp., including *P. fusca*, after repeated transfers also suggests that this character might not be stable (Vujanovic et al 2000).

Analysis of SSU sequence data indicates a phylogenetic affinity between *P. scopiformis*, *P. repens* and representatives of the Dothideales. These *Phialocephala* spp. species clearly are unrelated to other *Phialocephala* spp. studied and also are distinctly different from each other. The relatedness of *P. scopiformis*, *P. repens* and representatives of the Dothideales, as well as between *P. compacta* and representatives of the Hypocreales in this study, was not supported by the LSU data and remains unclear. Morphological evidence to support these affiliations also is lacking. Species characterized by brown conidiophores becoming paler toward the apex are not included in the Dothideales.

In this study we included an isolate of *Sporendocladia bactrospora*, a species that previously was accommodated in *Phialocephala*, as *P. bactrospora* (Kendrick 1961). Based on a study of conidiogenesis and the presence of ring-wall-building conidial development in this fungus, Wingfield et al (1987) transferred it to *Sporendocladia*. Conidial production through ring-wall building makes this fungus morphologically similar to *Thielaviopsis* anamorphs of *Ceratocystis*, in which conidia typically are produced in this manner (Nag Raj and Kendrick 1975, Paulin and Harrington 2000). Thus it was anticipated that the isolate of *S. bactrospora* included in this study would group together with *Ceratocystis* in the Microascales.

This study has enabled us to suggest appropriate phylogenetic placements for a number of *Phialocephala* spp., namely *P. dimorphospora*, *P. fortinii*, *P. scopiformis*, *P. repens*, *P. compacta*, *P. fusca* and *P. xalapensis*. Thus we confirm previous contentions that the genus is heterogeneous and that most species are unrelated. *Phialocephala* should be restricted to species that are similar to *P. dimorphospora*, based on sequence data, namely *P. fortinii*. Alternative generic names will be needed for other species.

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