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Phylogenetic relationships of *Chaetomium* and similar genera based on ribosomal DNA sequences

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Abstract: The 30 taxa sampled for this study were chosen based on their taxonomic position or particular morphological characters, which are related to those of *Chaetomium*. The 15 fungal isolates sequenced herein were obtained during a fungal biodiversity survey of tropical soils or from the collections of R.T. Hanlin. Fifteen species were selected from the published data. The aligned sequences of the 5′ portion of the 18S ribosomal DNA were analyzed to infer the phylogenetic relationships among *Chaetomium* and related genera. The sequence data confirmed the generic entity of *Achaetomium* and placed *Ascotricha* within the Xylariaceae. The analyses also defined the monophyletic lineage of the Microascaceae and the evolutionary linkages between the families Sordariaceae and Chaetomiaceae. The placement of *Coniochaeta* in the Coniochaetaceae and *Chaetomium* and *Achaetomium* in the Chaetomiaceae, under the order Sordariales, is supported by this study.

Key Words: *Achaetomium,* Ascomycota, *Ascotricha,* Chaetomiaceae, *Coniochaeta, Emericella nidulans, Eurotium rubrum, Kernia, Lophotrichus,* Microascales, *Petriella,* pyrenomycetes, *Sordaria,* Sordariales, *Thielavia,* Xylariales

INTRODUCTION

The genus *Chaetomium* Kunze consists of 300 described species of well-known cellulose degrading fungi. They are cosmopolitan in distribution and occur on a variety of substrata, including soil and dung, from the tropics to temperate regions.

Since the creation of the genus *Chaetomium* in 1817 and the introduction of the family Chaetomiaceae in 1885, many taxonomic studies have been conducted to arrange this group of fungi into a suitable classification scheme (Ames 1963, Arx et al 1986, Chivers 1915, Dreyfuss 1976, Seth 1970, Skolko and Groves 1953, Sörgel 1960). A major reason for disagreement on the taxonomic position of *Chaetomium* is the uncertainty of the relationship of this genus to other genera or families of pyrenomycetous fungi. This uncertainty is caused by the limited number of morphological and developmental characters that can be used in the separation of taxa. The nature of the ascomata, asci, and ascospores, the centrum structure, and perithecial habit are the main characters used as a basis for taxonomic separations, but these have not been enough to resolve problems in relationships and to determine the taxonomic position of *Chaetomium*.

From the mid-1980s molecular studies have been utilized in systematics to resolve problems posed by the limitation of morphological characters or in cases where morphological characters are in conflict, ambiguous, or missing (Bruns et al 1991, Hibbett 1992, Hillis 1987). Among them, DNA sequences encoding ribosomal RNA have been extensively employed in mycology to investigate phylogenetic relationships among organisms (Berbee and Taylor 1992a, b, c, Bryan et al 1995, Hausner et al 1993, Saenz et al 1994, Sherriff et al 1995, Spatafora and Blackwell 1993, 1994, Swann and Taylor 1993, Wingfield et al 1994). The use of nuclear small subunit ribosomal DNA (ss rDNA) sequence analyses has proven to be a suitable tool to infer evolutionary relationships among distantly related groups.

Phylogenetic studies of *Chaetomium* and related genera can reveal the evolutionary history and give the natural relationships among genera from which we can infer a reliable taxonomic system. Consequently, phylogenetic analyses using the ss rDNA have been conducted to assess relationships of *Chaetomium* and other related genera; the results of these studies are reported here.

MATERIALS AND METHODS

The 30 taxa sampled for this study were chosen based on their position in past and contemporary classifications and their possession of particular morphological characters (TABLE I). Among them, 15 isolates were sequenced by the authors and the other species were derived from previous studies (Berbee and Taylor 1992b, Melchers et al 1994, So-

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TABLE I. Fungal isolates used in this study

Name	GenBank	References
Achaetomium macrosporum Rai et al.	AF048785, AF048786	This study
Achaetomium strumarium Rai et al.	AF048787, AF048788	This study
Ascotricha xylina L. Ames	AF048789, AF048790	This study
Chaetomium aureum Chivers	AF048791, AF048792	This study
Chaetomium elatum Kunze	M83257	Berbee and Taylor (1992b)
Chaetomium funicola Cooke	AF048793, AF048794	This study
Chaetomium globosum Kunze	AF048795, AF048796	This study
Chaetomium nepalense (Udagawa & Sugiy.) v. Arx	AF048797, AF048798	This study
Coniochaeta sp.	AF048799, AF048800	This study
Coniochaeta tetraspora Cain	AF048813, AF048814	This study
Daldinia concentrica (Bolton : Fr.) Ces. & De Not.	U32402	Spatafora and Blackwell (1993)
Emericella nidulans (Eidam) Vuillemin	X78539	Melchers et al (1994)
Eurotium rubrum Kreig et al.	U00970	Berbee and Taylor (1993)
Hypocrea schweinitzii (Fr.) Sacc.	L36986	Spatafora and Blackwell (1993)
Hypomyces chrysospermus Tulas.	M89993	Berbee and Taylor (1992b)
Kernia nitida (Sacc.) Nieuwl.	AF048801, AF048802	This study
Lophotrichus indicus A.K. Saxena & Mukerji	AF048803, AF048804	This study
Lophotrichus plumbescens Morinaga et al.	AF048805, AF048806	This study
Microascus cirrosus Curzi	M89994	Berbee and Taylor (1992b)
Nectria cinnabarina (Tode : Fr.) Fr.	U32412	Spatafora and Blackwell (1993)
Neocosmospora vasinfecta E.F. Sm.	U32414	Spatafora and Blackwell (1993)
Neurospora crassa Schear & B.O. Dodge	X04971	Sogin et al (1986)
Petriella setifera (A. Schmidt) Curzi	AF048807, AF048808	This study
Petriella sordida (Zukal) G.L. Barron & J.C. Gilman	AF048809, AF048810	This study
Podospora anserina (Ces.) Niessl	X54864	Sogin et al (1986)
Pseudallescheria boydii (Shear) McGinnis et al.	M89782	Berbee and Taylor (1992b)
Sordaria fimicola (Roberge) Ces. & De Not.	X69851	Wilmotte et al (1993)
Sordaria humana (Fuckel) G. Winter	AF048811, AF048812	This study
Xylaria curta Fr.	U32417	Spatafora and Blackwell (1993)
Xylaria hypoxylon (L. : Fr.) Grev.	U20378	Spatafora and Blackwell (1993)

gin et al 1986, Spatafora and Blackwell 1993, Wilmotte et al 1993). One or more species from genera considered to be within the family were sequenced.

Fungal isolates were grown on either V8 agar or corn meal agar. Teleomorphs were produced by all isolates and were examined for the confirmation of species before harvesting. For DNA isolation, potato dextrose broth in 1.5 mL eppendorf tubes were inoculated with ascospores. These broth cultures were incubated at room temperature with shaking at 150 rpm for 48 to 72 h. The mycelium was harvested by centrifugation and washed twice with sterilized, double-distilled water. Total genomic DNA was extracted using the hexadecyltrimethylammonium bromide (CTAB) method (Graham et al 1994) or Lee and Taylor's extraction technique (Lee and Taylor 1990).

The partial small subunit rDNAs were amplified symmetrically using primers NS1 and NS6 (White et al 1990). Amplifications were performed using the Replicate-Pack Reagent Set (Boehringer Mannheim Co., Indianapolis, Indiana). Reactions were accomplished in a volume of 50 μL in the presence of 0.5 μM each of primers (NS1 and NS6) and 200 μM deoxynucleoside triphosphates in 1× reaction buffer containing 1.5 mM $MgCl_2$, 1.25U of *Taq* DNA polymerase with 2 μL of templates. The PCR-amplified DNA fragments of all species showed a single band product when examined on an 1% agarose gel.

The amplification reaction for ss rDNA was performed for 40 cycles with an initial denaturation of 2 min at 95 C and a final extension of 10 min at 72 C in a Perkin-Elmer-Cetus Thermal Cycler (Perkin-Elmer Co., Branchburg, New Jersey). Each cycle consisted of 30 s at 95 C for denaturation followed by 30 s at 55 C for annealing and 45 s at 72 C for extension.

The PCR products were purified from unincorporated nucleotides and primers using a Wizard DNA cleanup kit® (Promega Co., Madison, Wisconsin) following the manufacturer's protocol. Samples were concentrated to a final volume of 25 μL. Sequencing was performed on an automated DNA Sequencer 373A (Applied Biosystems, Weiterstadt, Germany) by the *Taq* DyeDeoxi Terminator™ cycle system.

The 15 newly sequenced partial ss rDNAs were aligned with other complete or nearly complete ascomycete sequences. The alignment was prepared in the PILEUP computer program (Genetics Computer Group, Madison, Wisconsin), which uses a simplification of the progressive alignment method of Feng and Doolittle (1987). Gaps were introduced to improve alignments and final alignments were optimized by hand. The alignment has been deposited in

TreeBase (http://herbaria.harvard.edu/treebase) as S335 and M438. Sequence alignments were compared and trees were generated with the PAUP (Phylogenetic Analysis Using Parsimony) program version 3.1 to distinguish the possible phylogenetic relationships among isolates (Swofford 1993). Due to the large number of taxa, only general heuristic searches were performed. Initial trees were obtained by stepwise addition in which taxa were connected in the simple algorithm. To improve the initial estimate branch swapping was executed with tree bisection reconnection algorithm. Rearrangements were tried a total of 158 226 times. A majority-rule consensus tree was produced from 100 bootstrap replications, and the percentage of times that a group of taxa appeared as a clade in the bootstrapped parsimony trees was obtained (Felsenstein 1985). Decay indices up to 5 steps were calculated as another assessment of the significance of internal branches in the cladogram (Donoghue et al 1992). Outgroup polarity was provided by the plectomycetes *Emericella nidulans* and *Eurotium rubrum.*

RESULTS

Amplification products with the primers NS1/NS6 obtained from 15 taxa showed the expected length of 1.4 kb. The data set from subsequent sequencing of the amplification products with the primers NS1/NS2 and NS3/NS4 consisted of sequences from the 5′ end of the 18S rDNA gene of approximately 1045 bp in length, representing about 60% of the total primary structure of ss rDNA. About 45 bp between the region of primers NS1 and NS3 and about 50 bp each of the 5′ end of NS1 and 3′ end of NS3 were excluded due to the unclear sequence data.

The total length of the sequence alignment was 958 positions. Of these, 132 (13.8%) sites were phylogenetically informative. To find the most likely tree topology, the data were analyzed by parsimony producing 18 equally most parsimonious trees of 331 steps, with consistency (CI) (Kluge and Farris 1969), retention (RI) (Farris 1989), and rescaled consistency indices of (RC) of 0.671, 0.847, and 0.568, respectively. One of the most parsimonious trees and a strict consensus tree are presented (FIGS. 1, 2, respectively).

The pyrenomycetous fungi included in this study formed a monophyletic clade with respect to the plectomycetous outgroups, *Em. nidulans* and *Eu. rubrum.* The ingroup consisted of three clades, the Xylariales, the Microascales/Nectriaceae/Hypocreaceae, and the Sordariales. The Microascales/Nectriaceae/Hypocreaceae and the Sordariales clades were supported by bootstrap values of 95% and 100%, respectively and a decay index of >5 steps.

The *Microascus* clade grouped with the *Nectria* clade, supported by a 57% bootstrap value, with the *Hypocrea* clade, including *Hypocrea* and *Hypomyces,* as a basal group. The *Nectria* clade encompassed two genera of the Nectriaceae, *Neocosmospora* and *Nectria.* The branch supporting this clade appeared in 96% of the bootstrap replicates and collapsed after four additional steps. The *Microascus* clade included five taxa sequenced in this study, two *Petriella* species, one *Kernia* species, and two *Lophotrichus* species, plus two published sequences, *Microascus cirrosus* and *Pseudallescheria boydii.* These fungi consistently clustered with 100% bootstrap support and a decay index of 5 steps.

The two *Coniochaeta* species possessing spores with germ slits formed a basal group to the *Sordaria* and the *Chaetomium* clades, which were supported as sister clades with a bootstrap confidence value of 98% and decay index of 4 steps. The *Sordaria* clade, including *Podospora, Neurospora,* and two *Sordaria* species, received only 61% bootstrap support. *Podospora* branched off earlier than *Neurospora,* followed by the two *Sordaria* species which were placed together with 89% bootstrap value. The *Chaetomium* clade contained four *Chaetomium* species representing morphological diversity within the genus and two *Achaetomium* species sequenced in this study, and another *Chaetomium* species from published data. This clade appeared 84 times out of 100 bootstrap replications and collapsed after two more evolutionary steps. *Chaetomium aureum* and *Ch. elatum* were grouped together with 63% bootstrap support. The *Achaetomium* species group received strong statistical support (100% bootstrap). Parsimony indicated a close relationship between the *Sordaria* and *Chaetomium* clades.

DISCUSSION

The classification of several of the genera included in this study, based on morphological characters, has been equivocal. Like most perithecial ascomycetes, *Chaetomium* was originally placed in the all-encompassing order Sphaeriales (Ainsworth 1961, 1971, Ainsworth and Bisby 1950, 1954, Alexopoulos 1952, Martin 1950). Subsequently, Alexopoulos (1962) recognized the order Chaetomiales for *Chaetomium* and related genera. This was based on the presence of perithecial hairs, the lack of paraphyses, and the evanescent asci. Whiteside (1961), however, reported that paraphyses are present in *Ch. globosum.* Alexopoulos and Mims (1979) placed *Chaetomium* in the order Xylariales, then Eriksson (1982) included the genus in the Sordariales, a placement followed by Hawksworth et al (1983) and most subsequent authors. Throughout these changes the genus has remained in the family Chaetomiaceae. The molecular data obtained in this study indicate a close relation-

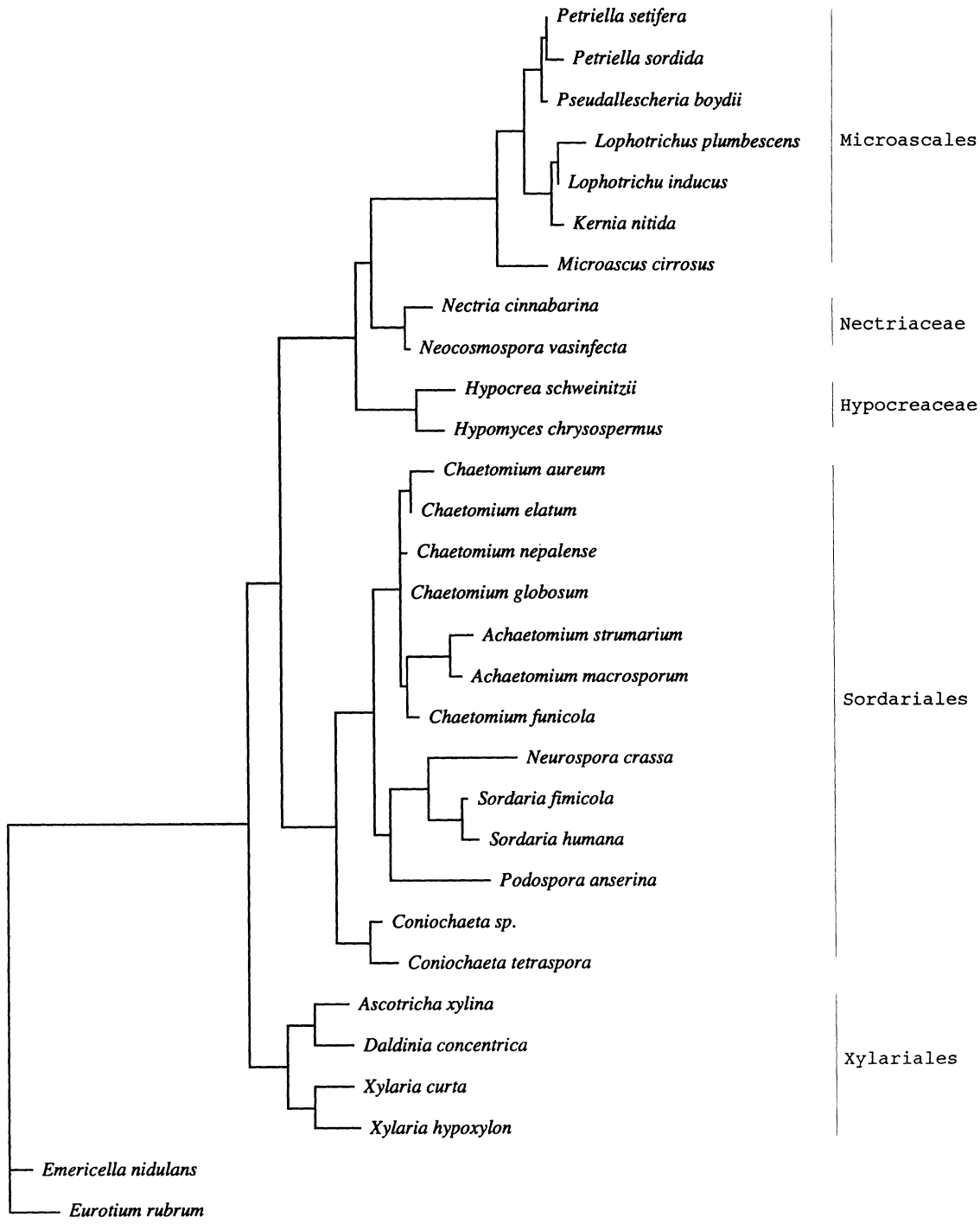

FIG. 1. One of 18 equally most parsimonious trees generated by PAUP program; 331 steps, CI = 0.671, RI = 0.847, RC = 0.568. The distance which corresponds to 10 base changes is indicated by a bar.

ship between the Chaetomiaceae and Sordariaceae, as was supported in other studies.

Achaetomium was established by Rai et al (1964) for taxa similar to *Chaetomium* but without the hairy ornamentations on the perithecia. Cannon (1986) suggested that *Achaetomium* might have been derived from the ancestor of the Sordariaceae rather than of the Chaetomiaceae because of the color of the ascospore, which is reminiscent of the former and unlike that of the latter. Rai et al (1970) proposed, and Mukerji and Saxena (1975) supported, the separate family Achaetomiaceae for *Achaetomium,* and Mukerji (1968) also proposed an independent order to accommodate the Achaetomiaceae. Because the separation of *Achaetomium* to the familial level made the *Chaetomium* clade polyphyletic, the inclusion of

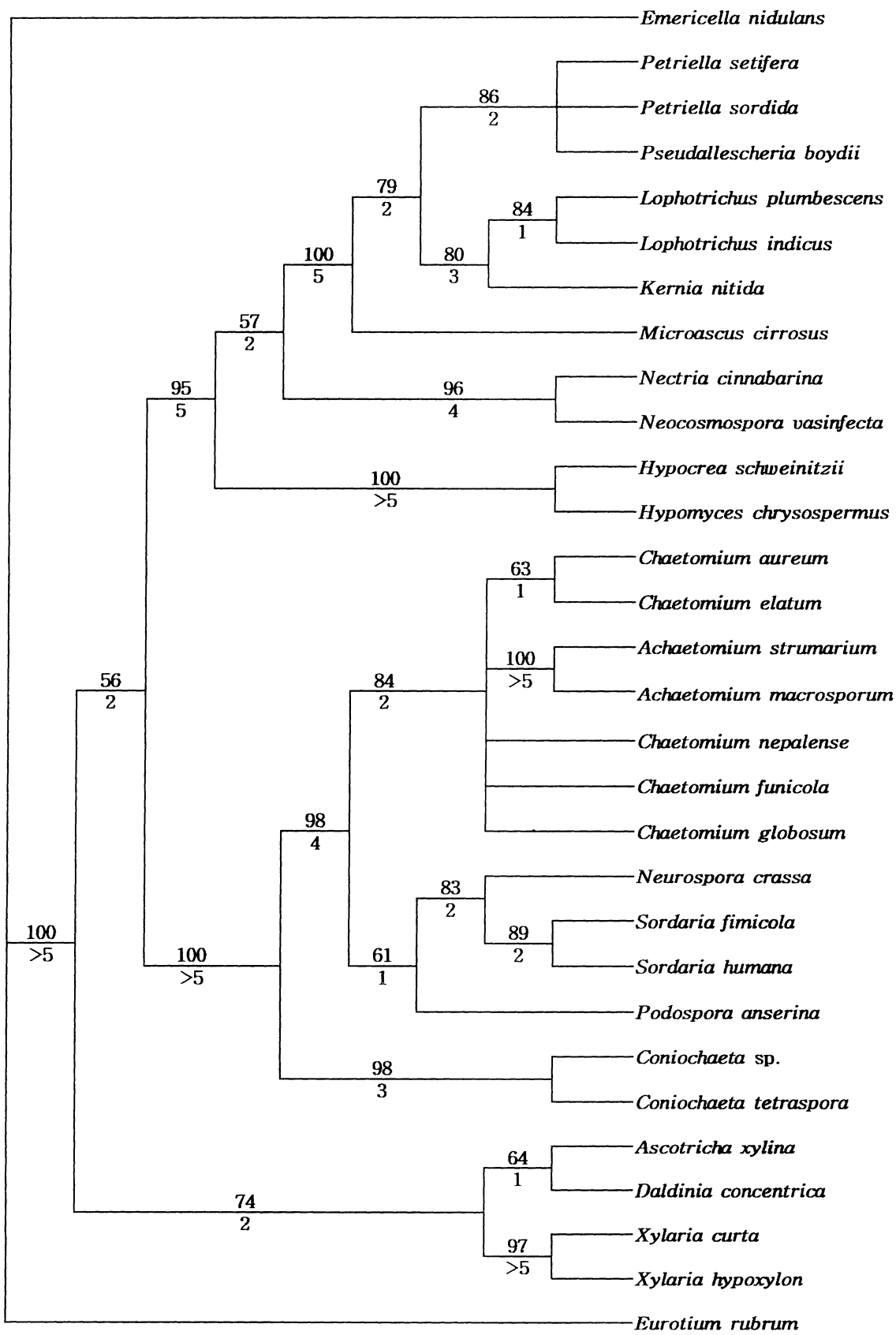

FIG. 2. Strict consensus of 18 most parsimonious trees. The numbers above and below the nodes indicate bootstrap values and decay indices, respectively.

Achaetomium as a member of the Chaetomiaceae is recommended based on molecular data presented here. The results showed that *Achaetomium* was derived from the ancestor of *Chaetomium,* contray to Cannon's hypothesis (FIGS. 1, 2).

Cannon (1986) proposed that *Ac. strumarium,* one of the two *Achaetomium* species included in this study, be transferred to the genus *Chaetomium.* The molecular data obtained, however, do not support such a transfer, as the two species of *Achaetomium* grouped together with 100% bootstrap support. The data also suggest that *Achaetomium* and *Chaetomium* share a common ancestor (FIG. 1).

Like *Chaetomium, Ascotricha* also has been placed in different orders. Although *Ascotricha* was considered to be closely related to *Chaetomium* due to its hairy ornamentations and nutritional habit (Ames 1963, Berkeley 1838, Kahn and Cain 1977, Mukerji 1968), the hairs of *Ascotricha* are geniculate, unlike those of *Chaetomium* species. Whiteside (1962a) showed that the filamentous hymenial paraphyses were better developed in *Ascotricha* than in *Ch. globosum.* Hawksworth (1971) suggested that *Ascotricha* was related to the xylariaceous fungi on the basis of ascospore shape and the prominent germ slit; the ascospores of *Chaetomium* possess germ pores. The close affinity between *Ascotricha* and species of the Xylariaceae (Alexopoulos et al 1996) is suggested by the centrum structure and the anamorphic states. The Xylariaceae have a filamentous centrum and blastosporous conidial state mostly in *Nodulisporium* and *Geniculosporium,* whereas the genus *Ascotricha* has filiform lateral and hymenial paraphyses in the young centrum (Whiteside 1962a), and an anamorph in *Dicyma,* in which conidia also are produced blastically (Hawksworth 1971). Læssøe (1994) excluded *Ascotricha* from the Xylariaceae because of its lack of a stroma, but Ju and Rogers (1996) included it in their key to genera in the family. The placement of the nonstromatic *Ascotricha* in the Xylariaceae is supported by the molecular data presented here (FIGS. 1, 2). The phylogenetic gene tree revealed that *Ascotricha* is a descendant of the common ancestor of xylariaceous fungi. The grouping of *Ascotricha* with *Daldinia* may be an artifact of the limited number of taxa employed in the analysis. The addition of more xylariaceous taxa to the analysis would better define the relationships and phylogeny of *Ascotricha* with other members of the Xylariaceae.

Benjamin (1949) included *Lophotrichus* in the Chaetomiaceae due to the well developed ascomatal hairs, but he also recognized the differences between the members of the Chaetomiaceae and *Lophotrichus,* such as the more or less submerged habit of the ascomata and the typical long necks of the latter. Whiteside (1962b) provided additional reasons which made a definite taxonomic placement of *Lophotrichus* difficult. Not only was the development of the ascocarp of *Lophotrichus* distinctly different from that occurring in *Chaetomium,* but there are differences in the centrum structure and the ascus as well. He pointed out the similarity of *Lophotrichus* to *Microascus* in centrum structure. Malloch (1970) also recognized the close resemblance of *Lophotrichus* to *Microascus longirostris* Zukal in general appearance, and transferred the genus *Lophotrichus* into the family Microascaceae. The ss rDNA sequence data are congruent with morphological and ontogenetic evidence observed by previous researchers, and indicate that *Lophotrichus* belongs to the *Microascus* clade with a high degree of confidence (FIG. 2). The data also suggest that it is the most recently evolved of the species examined. Although the Lophotricaceae was established in the Microascales by Seth (1971) to accommodate the genus *Lophotrichus* and its species, the retention of *Lophotrichus* as a member of the family Microascaceae (Alexopoulos et al 1996) is highly supported by rDNA sequence analyses. *Lophotrichus* were placed with other members of the Microascales in a clade with highly supported bootstrap value.

The genus *Petriella* develops evanescent asci at all levels within the centrum and releases its reddish-brown ascospores into the central cavity at maturity. *Petriella* has a *Graphium* conidial state which is annellidic and thus is very similar to *Scopulariopsis,* an anamorphic state of *Microascus.* Although some minor differences in ascogonium and ascogenous hyphae were observed by Corlett (1963, 1966), sequence data showed a close relationship between *Petriella* and *Microascus.* The mammalian pathogenic fungus, *Pseudallescheria* and two *Petriella* species collapsed into a branch supported by 86% bootstrap value.

Malloch and Cain (1971a) placed *Kernia* as a member of the Microascaceae sensu Malloch (1970), a placement supported by Alexopoulos et al (1996) and Hausner et al (1993), based on molecular data. This placement also is supported by the data obtained in the present study.

Coniochaeta was described as having asci that may or may not be evanescent, with a phialidic conidial state in *Phialophora,* and differing from the Sordariaceae in having ascospores with elongated germ slits, a characteristic of many xylariaceous fungi. Malloch and Cain (1971b) removed the genus *Coniochaeta* from the Sordariaceae to the Coniochaetaceae and suggested including *Ascotricha* in that family. If *Coniochaeta* is included as a member of the Sordariaceae the family shows polyphyletic traits, which is not desirable for taxonomic purposes. Accordingly the sep-

aration of *Coniochaeta* in the family Coniochaetaceae is strongly supported on the basis of the molecular data presented here. Although *Coniochaeta* groups with the *Sordaria* clade, it is intermediate morphologically in retaining the germ slit and well developed paraphyses of the Xylariales, but lacks the typical stroma and anamorph of this order.

In general, the molecular based relationships generated in this study were more or less consistent with species affinities that can be inferred from morphology, but the placement of some genera with ambiguous characteristics, including *Achaetomium, Ascotricha* and *Lophotrichus,* was resolved. The rDNA data broadened the original concept of the Chaetomiaceae, and supplemented morphological data derived from peridial wall structure, ascospores, asci, and ascomata, which by themselves were not sufficient to delimit the Chaetomiaceae.

Based on partial ss rDNA sequence data the genus *Chaetomium* in its present delimitation is a biological and phylogenetic entity that clearly belongs with the sordariaceous ascomycetes. It is a distinctive genus, as shown by its clear separation from the other genera included in this study. A common lineage for the Chaetomiaceae and the Sordariaceae also was statistically supported. The members of the *Sordaria* clade are closely related to those of the *Chaetomium* clade but are distinguished by having persistent asci with apical rings or thickenings and ascospores with gelatinous sheaths, cellular or gelatinous appendages, or ornamentations. Arx et al (1984) considered the ancestor of *Chaetomium* to be a member of the Sordariaceae with ostiolate ascomata and ascospore ejaculation. The phylogenetic tree generated in this study suggests that the families Chaetomiaceae and Sordariaceae are derived from the same common ancestor and diverged along different evolutionary pathways. The exclusion of *Ascotricha* from, and the inclusion of *Achaetomium* in the Chaetomiaceae indicates that ascomatal characters such as sterile hyphae or hairs appear to have been gained and/or lost several times during the evolution of members of the pyrenomycetes, perhaps in response to ecological conditions. Likewise, the separation of *Ascotricha* and *Coniochaeta,* both of which possess ascospores with germ slits, indicates that this character has been lost more than once during evolution and that, in itself, this is not a definitive taxonomic character.

The 13 isolates of sordariaceous fungi studied formed a monophyletic taxonomic group, the Sordariales, which branched with high bootstrap support (100%) to form distinct subgroups (FIG. 2). These subgroups correspond to the families Coniochaetaceae, Sordariaceae, and Chaetomiaceae.

A second well defined clade is the Microascales clade, with the single family Microascaceae. Arx et al (1984) once restricted the Microascaceae to the genera *Microascus* and *Kernia,* which have small, roundish or ovate, often catenulate asci and small ascospores with a single germ pore. The expanded familial concept of the Microascaceae as characterized by Alexopoulos et al (1996), however, is highly supported based on rDNA sequence data which indicate a monophyletic lineage for the microascaceous fungi. *Microascus,* which has irregularly disposed asci and blastic annellosporous amerospores such as *Scopulariopsis* or didymosporous aleuriospores such as *Wardomyces,* appears basal to the remaining members of the Microascaceae. The Microascales clade appears as a sister group to the Hypocreales clade, but the long branch with 23 evolutionary steps (FIG. 1) in the Microascaceae clade suggests that the fungi in the clade have diverged significantly from the Hypocreales. The results described herein are consistent with those reported earlier (Spatafora and Blackwell 1993, 1994) and they provide data on the relationships of additional genera of pyrenomycetes.

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