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Phylogenetic placement of anamorphic species of *Chalara* among *Ceratocystis* species and other ascomycetes

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Abstract: *Ceratocystis sensu stricto* is a genus of plant-pathogenic pyrenomycetes including species with ascospores dispersed by insects. All known species of *Ceratocystis* have *Chalara* anamorphs. However, chalara-like anamorphs have been connected to other genera of ascomycetes, and not all *Chalara* species have known teleomorphs. Portions of the 18S and 28S rDNA domains were sequenced and aligned with those of other ascomycetes, and parsimony analysis was used to determine which *Chalara* species have phylogenetic affinities with *Ceratocystis*. Twenty-three *Chalara* species lacking known teleomorphs fell into three major groups. Sixteen species appeared related to the *Leotiales* (discomycetes), including *Cyathicula coronata* and *C. strobilina,* which have *Chalara* anamorphs. *Chalara hyalina* was the only species allied with the loculoascomycetes. Six *Chalara* species, all plant pathogens, were placed with *Ceratocystis* in a strongly supported clade. Two of these *Chalara* species, *Ch. australis* and *Ch. neocaledoniae*, form *Ceratocystis* perithecia and nonviable ascospores when mated with other *Ceratocystis* species in interspecific crosses. The four other *Chalara* species (*Ch. ovoidea*, *Ch. thielavioides*, *Ch. populi*, and *Ch. elegans*) have no known teleomorphs, but all form aleurioconidia typical of the anamorph genus *Thielaviopsis* and may represent an asexual lineage within *Ceratocystis*.

Key words: SSU rDNA, LSU rDNA, *Microascales*, *Thielaviopsis*, *Leotiales*, *Cyttaria*, *Cryptendoxyla*, systematics.

Introduction

Our studies focus on the systematics and biology of the pyrenomycete genus *Ceratocystis* Ellis & Halstead *sensu stricto*, a monophyletic group of plant-pathogenic fungi, including *C. fimbriata* and *C. fagacearum* (Witthuhn *et al.*, 1999), which are dispersed by insects. In the present study, we were interested primarily in identifying anamorphic species with *Ceratocystis* affinities. All *Ceratocystis* species have *Chalara* anamorphs, which are characterized by deep-seated phialides and conidia produced by ring-wall building (Minter, 1982, 1983; Nag Raj & Kendrick, 1975, 1993). For instance, *Chalara ungeri* Sacc. is the anamorph of *Ceratocystis coerulescens* (Münch) Bakshi (Harrington & Wingfield, 1998), and *Ch. quercina* Henry is the anamorph of *Ceratocystis fagacearum* (Nag Raj & Kendrick, 1975). Two *Chalara* species, *Ch. australis* and *Ch. neocaledoniae*, are clearly related to *Ceratocystis* on the basis of isozyme electromorphs, rDNA sequences, *MAT-2* DNA sequences, and partial interfertility (formation of sterile perithecia) with *Ceratocystis* species (Harrington *et al.*, 1996; Harrington *et al.*, 1998; Harrington & McNew, 1998; Witthuhn *et al.*, 1999; Witthuhn *et al.*, 2000).

Other *Chalara* species may also have *Ceratocystis* affinities, although *Chalara* anamorphs have been found in many orders of ascomycetes, especially the *Leotiales* (Table 1). The type species of *Chalara* is *Ch. fusidioides* (Corda) Rabenh., a saprobe occurring on *Fragaria vesca*, *Pinus* sp., *Podocarpus hallii*, *Vitis* sp., and pseudoperithecia of *Mycosphaerella* (Nag Raj & Kendrick, 1993), but no teleomorph is known for this species. Undescribed species of the Leotialean genera *Lanzia*, *Cyathicula* and *Volutaria* have been reported to have chalara-like anamorphs (Hennebert & Bellèmere, 1977; Gams & Philippi, 1992). Chalara-like anamorphs are also found in the

Table 1. Teleomorph species reported to have chalara-like anamorphs

Order	Teleomorph	Anamorph	References
Microascales	All species of *Ceratocystis*	*Chalara* spp.	Halsted, 1890; Nag Raj & Kendrick, 1975; Harrington, 1981, 1987; Hausner & Reid, 1993
	Ceratocystiopsis falcata Wright & Cain	*Chalara* sp.	Rayner & Hudson, 1977; Upadhyay, 1981; Hutchinson & Reid, 1988; Nag Raj & Kendrick, 1993; Seifert *et al.*, 1993
	Sphaeronaemella raphani Malloch	*Chalara* sp.	Malloch, 1974
Laboulbeniales	*Pyxidiophora asterophorae* (Tul.) Lindau	*Chalara* sp.	Müller & von Arx, 1973; Blackwell & Malloch, 1989
	P. arvernensis (Bret. & Faur.) Lundq.	*Chalara* sp.	Lundqvist, 1980; Blackwell & Malloch, 1989
	P. grovei Lundq.	*Chalara* sp.	Lundqvist, 1980; Blackwell & Malloch, 1989
Leotiales	*Allophylaria fide* Graddon	*Chalara* sp.	Graddon, 1980
	Belonidium albidum Grelet & Crozals	*Chalara* sp.	Grelet, 1951; Berthet, 1964
	Bioscypha cyatheae Sydow	*Chalara* sp.	Samuels & Rogerson, 1990
	B. pteridicola Samuels & Rogerson	*Chalara* sp.	Samuels & Rogerson, 1990
	Bisporella sulfurina (Quél.) Boud.	*Bloxamia truncata* Berk. & Br. (sporodochia with chalara-like conidiophores)	Berthet, 1964; Nag Raj & Kendrick, 1975
	Calycellina carolinensis Nag Raj & W.B. Kendr.	*Chaetochalara aspera* (Pirozynski & Hodges) P.M. Kirk	Nag Raj & Kendrick, 1975; Farr *et al.*, 1989
	Chlorociboria aeruginascens (Nylander) Kanouse ex Ramamurthi *et al.*	*Dothiorina tulasnei* (Sacc.) Höhnel (coelomycetous with chalara-like conidiophores)	Dixon, 1975; Nag Raj & Kendrick, 1993
	Cyathicula coronata (Bull.) De Not.	*Chalara coronata* (Bull.) De Not.	Dennis, 1956
	Cyathicula strobilina (Fr.) Korf & Dixon	*Chalara strobilina* Sacc.	Philippi, 1984; Gams & Philippi, 1992
	Pezizella vulgaris (Fr.) Sacc.	*Chalara cylindrica* P. Karsten	Farr *et al.*, 1989
	Phaeoscypha cladii (Nag Raj & W.B. Kendr.) Spooner	*Chalara cladii* M.B.Ellis	Nag Raj & Kendrick, 1975; Kendrick, 1980; Kirk & Spooner, 1984; Nag Raj & Kendrick, 1993
Trichosphaeriales	*Ascochalara gabretae* Réblová	*Chalara* sp.	Réblová, 1999
	Chaetosphaeria bramleyi C. Booth	*Chalara* sp.	Gams & Holubová-Jechová, 1976
	Chaetosphaeria chalaroides Hol.-Jech.	*Chalara breviclavata* Nag Raj & W.B. Kendr.	Gams & Holubová-Jechová, 1976; Holubová-Jechová, 1984
	Melanochaeta aoteorae (S. Hughes) E. Müller *et al.*	*Chalara* sp.	Müller *et al.*, 1969; Nag Raj & Kendrick, 1975; Müller & Samuels, 1982
Sordariales	*Cryptendoxyla hypophloia* Malloch & Cain	*Chalara* sp.	Malloch & Cain, 1970; Suh & Blackwell, 1999
Dothideales	*Quasiconcha reticulata* M. Barr & Blackwell	*Chalara* sp.	Barr & Blackwell, 1980; Blackwell & Gilbertson, 1985

Laboulbeniales, *Trichosphaeriales*, *Dothideales*, *Sordariales*, and *Microascales* (Table 1).

Morphological criteria to correlate *Chalara* anamorphs with ascomycete orders are not apparent (Gams & Philippi, 1992; Nag Raj & Kendrick, 1993), so we relied on parsimony analysis of rDNA sequences to identify those *Chalara* species with *Ceratocystis* affinities. We sequenced strains representing 23 species of *Chalara* (Table 2) with no known teleomorph that were obtained from the Centraalbureau voor Schimmelcultures or from our own collection. Some other ascomycetes were included for comparison (Table 3).

Materials and methods

Strains and culture conditions

All strains of *Chalara* and *Ceratocystis* were examined microscopically to confirm the presence of the *Chalara* anamorph. Strains were grown at room temperature on malt-yeast extract agar (0.2% Difco yeast extract, 2% Difco malt extract, 2% Sigma agar) for 2 to 3 weeks prior to DNA extraction.

Polymerase chain reaction

Template DNA for PCR was obtained either directly by scraping the fungal mycelium without extracting DNA (Harrington & Wingfield, 1995) or by extracting DNA (DeScenzo & Harrington, 1994). Extracted DNA was used only when the scraping method failed. For DNA extraction, strains were grown at room temperature (approximately 21°C) in 30 ml of broth medium (0.2% yeast extract, 2% malt extract) for 2 to 6 weeks.

Portions of the small subunit (18S) and large subunit (28S) of the nuclear ribosomal DNA (rDNA) were amplified and sequenced. The primers used for PCR amplification and DNA sequencing are listed in Table 5. The primer pairs SR9R/ITS2 and SR10R/NS8 were used for amplification and sequencing, respectively, of the small subunit rDNA. The primer pairs LROR/LR5 and LROR/LR3 were used for amplification and sequencing, respectively, of the large subunit rDNA.

The 100 ml amplification reactions included 4 mM $MgCl_2$; 5% DMSO; 1 X Sigma buffer; 200 mM dNTPs; 0.5 mM of the forward and reverse primers; 3 units Taq polymerase (Sigma Chemical Co, St. Louis, MO, USA), and 10 to 40 ng of extracted DNA or mycelial scrapes. Cycling conditions (MJ Research, Inc. thermocycler; PTC-100) for LSU amplification were an initial denaturation at 94°C for 95 s followed by 35 cycles of denaturation (94°C) for 35 s, annealing at 49°C for 60 s, and extension at 72°C for 180 s. Final extension was at 72°C for 15 min. Cycling conditions for amplification of the SSU were as described for the LSU except that the annealing temperature was 42°C. The PCR amplification product was purified using a QUIAquick DNA Purification Kit (Quiagen, Hilden, Germany) and quantified on a TKO 100 mini-fluorometer. Automated sequencing was performed at the Iowa State University DNA Sequencing and Synthesis Facility using the Applied Biosystems (Foster City, CA) Prism BigDye terminator cycle sequencing kit with AmpliTaq DNA polymerase FS on an ABI PRISM 377 DNA Sequencer (Perkin-Elmer, USA).

Analysis

Sequences were manually aligned by inserting gaps, but ambiguously aligned characters were eliminated before parsimony analysis (PAUP 4.0b3a, Swofford, 1998). Gaps were treated as a fifth character in the LSU data set, while gaps were treated as missing data in the SSU analyses. Only parsimony informative sites were used in the phylogenetic analyses. Maximum parsimony heuristic searches were performed with all characters having equal weight. Robustness of the internal branches of the tree was evaluated by 1000 bootstrap replications using heuristic searches. Trees were rooted at the internal node with basal polytomy, using basidiomycetes as outgroup taxa. The homogeneity of LSU and SSU data sets was tested using the partition homogeneity test in PAUP 4.0b3a (Farris *et al.*, 1995).

Results

Partial sequences of the SSU gene were obtained from 14 species of *Chalara* with no known teleomorph. Using BLAST searches (2.0, National Center for Biotechnology Information, Bethesda, Maryland), 13 of the 14 sequences were nearest to SSU sequences of either *Ceratocystis* species or members of the *Leotiales*. The BLAST search with the SSU sequence of *Ch. hyalina* showed it to be nearest the SSU sequences of *Mycospharella mycopappi*, *Herpotrichia juniperi*, and other loculoascomycetes.

The SSU sequences of *Ch. hyalina* and loculoascomycete species (*Chaetothyriales*, *Dothideales* and *Pleosporales* shown in Table 4) were excluded from the analysis because their sequences did not group with those of the other *Chalara* species, and trees that included *Ch. hyalina* and the loculoascomycetes did not resolve well. Similarly, sequences of some members of the *Eurotiales* (*Monascus purpureus*, *Eremascus albus*, and the anamorphic *Paecilomyces variotii*) grouped near each other but not to the SSU sequences of the *Chalara* species or the other ascomycetes studied; these sequences (Tables 3 and 4) were also excluded from the SSU analysis. Sequences of *Byssoascus*, *Myxotrichum*, and *Pseudogymnoascus* (*Onygenales*, *Myxotrichaceae*) and *Erysiphales* were similar to those of the *Leotiales*, but inclusion of these sequences (Tables 3 and 4) greatly increased the number of most par-

Table 2. Sequenced strains of *Chalara* species investigated here and GenBank accession numbers.

Species	Strain number[a]	GenBank SSU rDNA	GenBank LSU rDNA
Ch. affinis Sacc. & Berl.	CBS 562.77 (1)	–	AF222446
	CBS 620.75 (2)	–	AF222447
Ch. angustata Kawalski & Halmschlager	CBS 231.96	–	AF222448
Ch. aurea (Corda) S. Hughes	CBS 729.69	AF222503	AF222449
Ch. australis Kile	C 448	AF222504	AF222450
Ch. austriaca Fautr. & Lamb.	CBS 264.94	AF222505	AF222451
Ch. brevispora Nag Raj & W.B. Kendr.	CBS 595.94	–	AF222452
Ch. constricta Nag Raj & W.B. Kendr.	CBS 248.76 (1)	–	AF222453
	CBS 731.92 (2)	AF222506	AF222454
Ch. crassipes (Preuss) Sacc.	CBS 829.71 (1)	–	AF222455
	CBS 216.84 (2)	–	AF222456
Ch. cylindrosperma P. Karst.	CBS 658.79	AF222507	AF222457
Ch. elegans Nag Raj & W.B. Kendr.	C 853 (1)	AF222508	AF222458
	CBS 414.52 (2)	–	AF222459
Ch. ellisii Nag Raj & W.B. Kendr.	CBS 928.97	–	AF222460
Ch. fungorum (Sacc.) Sacc.	CBS 942.72 (1)	–	AF222461
	CBS 240.82 (2)	–	AF222462
Ch. hyalina Morgan-Jones & Gintis	CBS 558.92	AF222509	AF222463
Ch. kendrickii Nag Raj	CBS 490.77	–	AF222464
Ch. longipes (Preuss) Cooke	CBS 875.85 (1)	–	AF222465
	CBS 411.76(2)	–	AF222466
Ch. microchona W. Gams	CBS 867.73 (1)	AF222510	AF222467
	CBS 121.74 (2)	–	AF222468
Ch. microspora (Corda) S. Hughes	CBS 131.74 (1)	–	AF222469
	CBS 261.75 (2)	–	AF222470
Ch. neocaledoniae Dadant ex Kiffer & Delon	CBS 149.83	AF222511	AF222471
Ch. ovoidea Nag Raj & W.B. Kendr.	CBS 136.88	AF222512	AF222472
Ch. parvispora Nag Raj & S. Hughes	CBS 983.73 (1)	–	AF222473
	CBS 385.94 (2)	–	AF222474
Ch. populi Veldeman ex Kiffer & Delon	CBS 484.71	AF222513	AF222475
Ch. sessilis Nag Raj & W.B. Kendr.	CBS 405.81	AF222514	AF222476
Ch. thielavioides (Peyr.) Nag Raj & W.B. Kendr.	C 1362 (1)	AF222517	AF222479
	CBS 130.39 (2)	AF222518	AF222480

[a] C = Culture collection of T.C. Harrington; CBS = Centraalbureau voor Schimmelcultures, Baarn, Netherlands; ATCC = American Type Culture Collection, USA. Numbers in parenthesis indicate more than one strain of a species sequenced.

Table 3. Sequenced strains of other genera investigated here and GenBank accession numbers

Order	Name	Strain number[a]	GenBank[b] SSU rDNA	GenBank[b] LSU rDNA
Erysiphales	*Blumeria graminis* (DC.) Speer f. sp. *hordei*	from *Hordeum*[c]	AF222534*	AF222494*
Eurotiales	*Eremascus albus* Eidam	CBS 975.69	AF222536*	–
	Monascus purpureus Went	CBS 281.34	–	AF222496*
anam.*Eurotiales*	*Paecilomyces variotii* Bainier	CBS 284.48	–	AF222501*
Leotiales	*Cyathicula coronata* (Bulliard : Fr.) De Not.	CBS 197.62	AF222532	AF222491
	Cyathicula strobilina (Fr. : Fr) Korf & Dixon	CBS 803.84 (1)	AF222515	AF222477
		CBS 643.85 (2)	AF222516*	AF222478*
	Neobulgaria pura (Fr. : Fr) Petrak	CBS 477.97	AF222533	AF222492
anam.*Leotiales*	*Phialophora gregata* (Allington & D.W.Chamb.) W.Gams	P 283	AF222526	AF22502
Microascales	*Ceratocystis adiposa* (Butler) Moreau	C 998, CBS 600.74	AF222519	AF222481
	Ceratocystis eucalypti Yuan & Kile	C 457	–	AF222482
	Ceratocystis fagacearum (Bretz) Hunt	C 1305	AF222520	AF222483*
	Ceratocystis fimbriata Ellis & Halstead	C 1390, IFO 30501 (1)	AF222521	AF222484
		C 1393, IFO 32969 (2)	AF222527*	AF222485*
		C 1004, CBS 153.62 (3)	–	AF222486*
	Ceratocystis moniliformis (Hedgecock) Moreau	C 1007, CBS 204.90	AF222528	AF222487*
	Ceratocystis paradoxa (Dade) Moreau	C 1021	AF222529	AF222488
	Ceratocystis virescens (Davidson) Moreau	C 74	AF222530	AF222489*
Onygenales	*Byssoascus striatosporus* (Barron & C.Booth) von Arx (*Myxotrichaceae*)	CBS 642.66	AF222535*	AF222495
Phyllachorales	*Plectosphaerella cucumerina* (Lindf.) W. Gams	P 16	AF222522	AF222497*
	Glomerella cingulata (Stoneman) Spauld. & H. Schrenk	AG1S1	AF222531	AF222490
Sordariales	*Cryptendoxyla hypophloia* Malloch & Cain	C 1531, FR 58	AF222524	AF222499

[a] CBS = Centraalbureau voor Schimmelcultures, Netherlands; A, C, and P = Culture collection of T.C. Harrington; FR = Ontario Ministry of Health; IFO = Institute for Fermentation, Osaka, Japan

[b] * Sequences used for comparison and deposited in GenBank, but not included in phylogenetic trees

[c] From a barley leaf maintained in a greenhouse at Iowa State University

simonious trees, and these sequences were excluded from the final analysis of the SSU data.

Nine out of 563 SSU characters, including gaps, were ambiguously aligned and were therefore eliminated from the data set. Fifty equally most parsimonious trees of 359 steps were derived from analysis of the 98 phylogenetically informative positions. The consistency (CI), homoplasy (HI), retention (RI), and rescaled consistency (RC) indices were 0.5376, 0.4624, 0.8021, and 0.4312, respectively.

Analysis of the SSU sequences placed most of the *Chalara* species into two groups: the *Leotiales* or the genus *Ceratocystis* (Fig. 1). The *Leotiales* clade inferred by the SSU analysis was only weakly supported by bootstrap analysis and included *Cyttaria darwinii* (*Cyttariales*) and the anamorphic species *Phialophora gregata*. Two members of the *Leotiales* with known *Chalara* anamorphs (*Cyathicula coronata* and *C. strobilina*) were grouped near six *Chalara* species with no known teleomorph. Six other *Chalara* species had SSU sequences identical to *Ceratocystis fimbriata* or *C. virescens* (Fig. 1). The *Ceratocystis* clade inferred by the SSU analysis was strongly supported by bootstrap analysis, but there was no support for the clade including *Ceratocystis* and the *Microascales*.

Partial sequences of the LSU gene were obtained from 23 species of *Chalara* with no known teleomorph. As with the SSU sequences, all but one of the 23 species showed LSU sequences similar to those of either the *Leotiales* or *Ceratocystis* based on BLAST searches. The LSU sequence of *Ch. hyalina* was distinct from that of the other *Chalara* species, with a BLAST search indicating a relatively low relationship to some species of loculoascomycetes (such as *Aureobasidium pullulans* and *Mycosphaerella mycopappi*, Table 4) and to some species in the *Leotiales* clade (Fig. 2). The sequences of *Ch. hyalina*, *Chaetothyriales*, *Dothideales*, *Pleosporales*, *Erysiphales*, *Eurotiales*, *Halosphaeriales*, *Ophiostomatales*, and *Xylariales* (Tables 3 and 4) were excluded from the final LSU analyses because they did not group with the other *Chalara* species.

One hundred and twenty-seven out of 568 LSU characters, including gaps, were ambiguously aligned and, therefore, eliminated before analysis. One hundred and seven equally most parsimonious trees of 542 steps were derived from analysis of 123 phylogenetically informative positions. The CI, HI, RI, and RC values were 0.5314, 0.4686, 0.7976, and 0.4238, respectively.

As with the SSU analysis, the LSU sequence analysis placed most of the *Chalara* species into either the *Ceratocystis* clade or into the *Leotiales* (Fig. 2). The *Leotiales* clade inferred by the LSU data had 71% bootstrap support, and the clade containing the orders of pyrenomycetes had strong (99%) support. The *Ceratocystis* clade grouped with the *Hypocreales*, *Phyllachorales*, and two genera of the *Microascales*. Included in the *Ceratocystis* clade were six *Chalara* species, two (*Ch. australis* and *Ch. neocaledoniae*) with the same LSU sequence as *Ceratocystis eucalypti*, and four (*Ch. elegans*, *Ch. populi*, *Ch. ovoidea*, and *Ch. thielavioides*) with LSU sequences similar to those of *C. paradoxa* and *C. adiposa* (Fig. 2).

The partition homogeneity test of the combined SSU and LSU data sets showed that the Type 1 error rate (tail probability) for rejecting the null hypothesis was $p = 0.03$, indicating incongruence of the two data sets.

Discussion

The genus *Chalara*, as currently recognized, is a large and morphologically diverse group (Nag Raj & Kendrick, 1993), and it is not surprising that the rDNA sequence analyses showed the group to be polyphyletic. *Chalara* species with no known teleomorph fell into three groups. *Chalara hyalina* was distinct, perhaps with affinities to loculoascomycetes. Sixteen of the studied *Chalara* species had rDNA sequences similar to those of the *Leotiales*. Six species of *Chalara* were placed clearly in the *Ceratocystis* clade that was inferred by both SSU and LSU sequences.

We were unable to obtain strains of all described species of *Chalara*, so it is likely that there are *Chalara* species with affinities to other ascomycetous groups. Nag Raj & Kendrick (1975) recognized 58 species of *Chalara* with no known teleomorphs and grouped these based on spore septation. *Chalara kendrickii* and *Ch. aurea* are representatives of the didymoconidia group, and all other *Chalara* species that we studied had non-septate phialoconidia. No representatives of the species with dictyoconidia or phragmoconidia were available, and we did not study species with chalara-like conidiophores in sporodochia or synnemata, e.g., *Chalarodendron* C.J.K. Wang & B. Sutton (Wang & Sutton, 1984). Numerous other chalara-like genera (Nag Raj & Kendrick, 1975; Gams & Holubová-Jechová, 1976) await phylogenetic study.

Chalara hyalina has a relatively fast growth rate when compared to the *Chalara* species with affinities to the *Leotiales*. Furthermore, the SSU and LSU sequences of *Ch. hyalina* were similar to those of loculoascomycetes and differed from all other *Chalara*

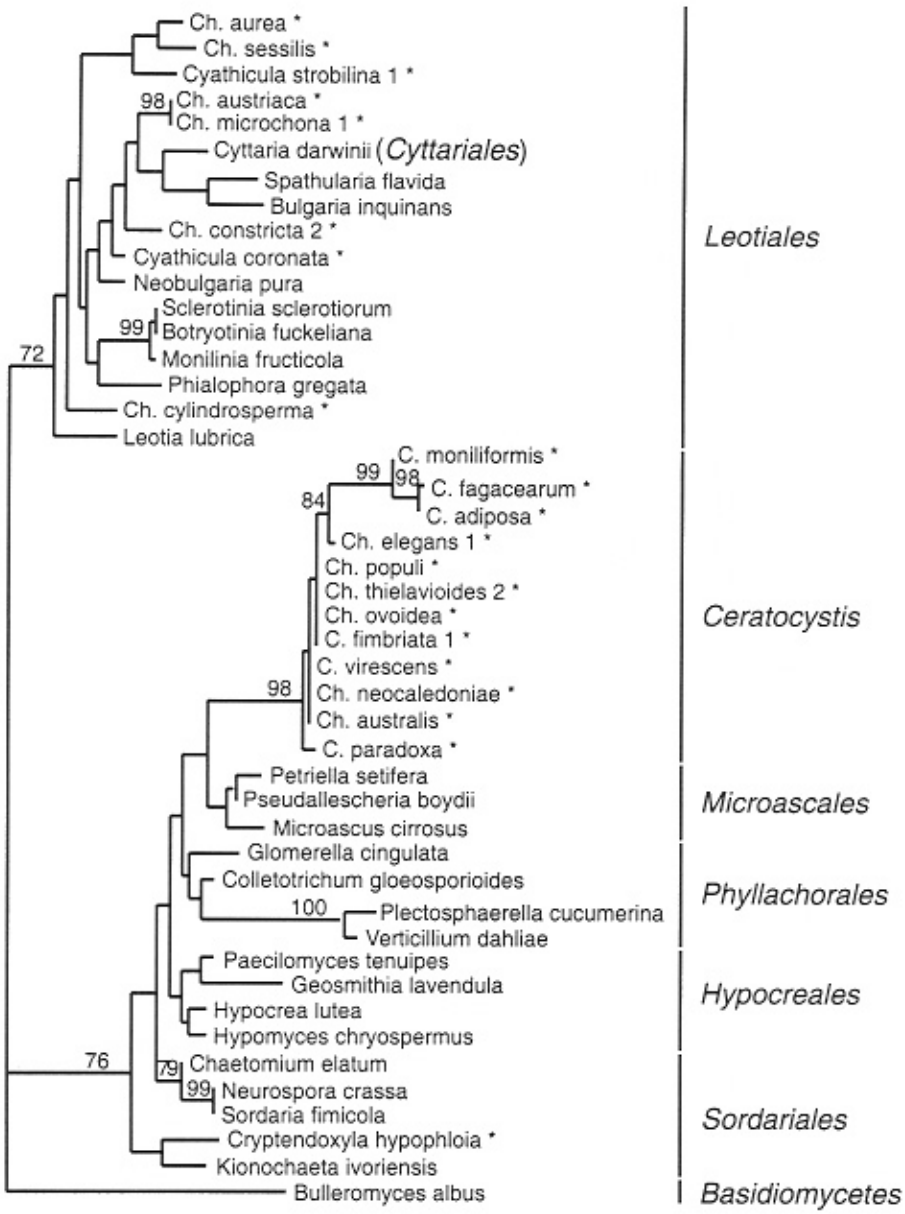

Fig. 1. One of 50 most parsimonious trees based on a portion of the 18S (small subunit) rDNA of *Chalara* species and ascomycete representatives. Asterisks after the taxon labels indicate *Chalara* species or teleomorphic species with chalara-like anamorphs. Bootstrap values greater than 50% are indicated above the branches. The tree is rooted to the basidiomycete *Bulleromyces albus*.

species studied. One loculoascomycete, *Quasiconcha reticulata*, is reported to have a *Chalara* anamorph (Blackwell & Gilbertson, 1985), but no culture of this species was available for our study. *Chalara hyalina* resembles the anamorph of *Cryptendoxyla hypophloia* and *Ch. microspora*, although *Ch. microspora* has pigmented conidiophores, and its conidia are smaller than those of *Ch. hyalina* (Morgan-Jones *et al.*, 1984). *Chalara microspora* has an LSU rDNA sequence that places it in the *Leotiales*, and *Cryptendoxyla hypophloia* has rDNA sequences that suggest a relationship to the *Sordariales*.

Parsimony analyses comparing the rDNA sequences of *Ch. hyalina* to the *Chaetothyriales*, *Dothideales* and *Pleosporales* (data not shown) were inconclusive in placing *Ch. hyalina*. The SSU analysis showed little affinity of *Ch. hyalina* to these loculoascomycetes, but the LSU analysis placed *Ch. hyalina* near the *Pleosporales*.

Sixteen of the *Chalara* species had rDNA sequences that suggest a phylogenetic relationship with the *Leotiales*, an order with a number of reported *Chalara* anamorphs. Relationships among these *Chalara* species and among the *Leotiales* were not resolved by these data. Two strains identified as identical *Chalara* species were commonly shown to have different rDNA sequences. Such discrepancies were seen between LSU sequences of two strains of *Ch. microspora*, *Ch. micro-*

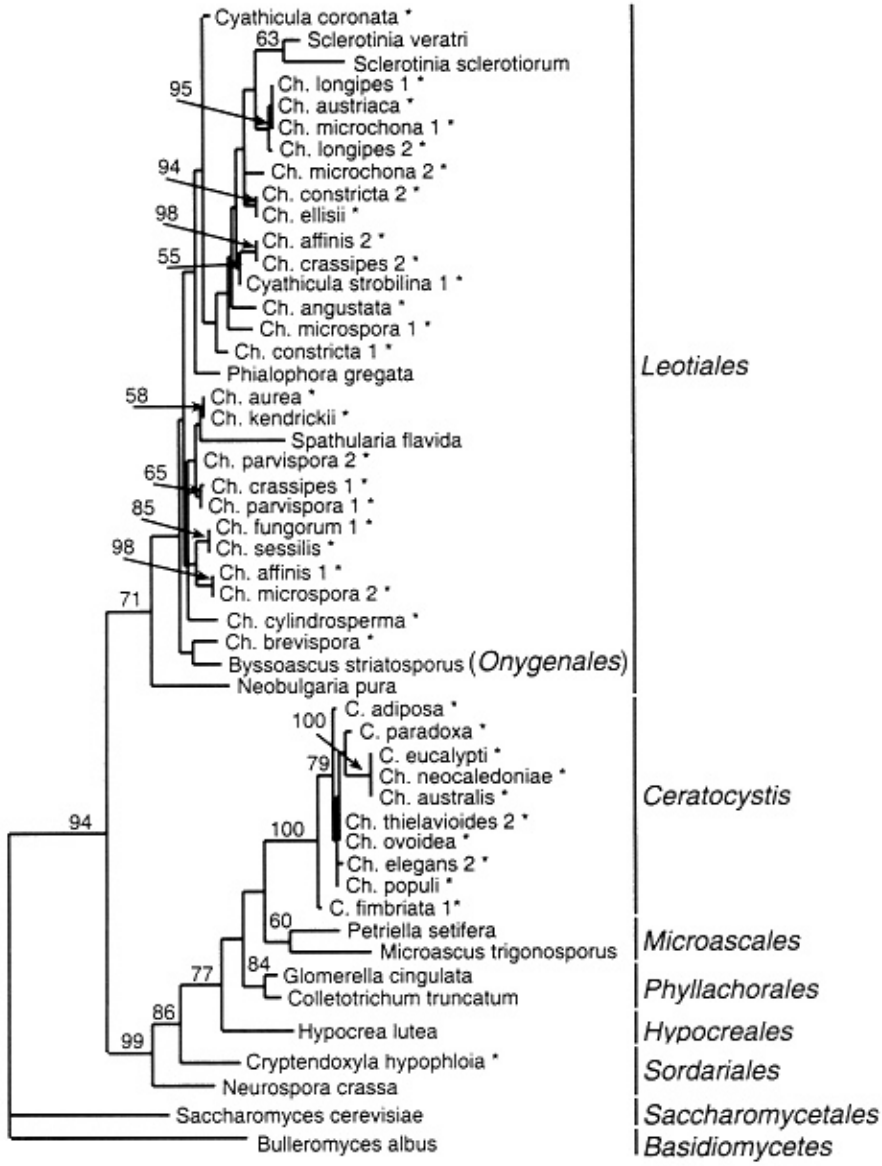

Fig. 2. One of 107 most parsimonious trees based on a portion of the 28S (large subunit) rDNA of *Chalara* species and ascomycete representatives. Asterisks after the taxon labels indicate *Chalara* species or teleomorphic species with chalara-like anamorphs. Bootstrap values greater than 50% are indicated above the branches. The tree is rooted to the basidiomycete *Bulleromyces albus*.

chona, *Ch. constricta*, *Ch. crassipes*, and *Ch. affinis*. The species determinations for many of these strains may be in error, but we confirmed the presence of chalara-like conidiophores in all of these cultures.

Cyttaria has been placed in the order *Cyttariales*, but it has been suggested that it has affinities to the *Leotiales* (Mengoni, 1989; Gamundí, 1991). *Cyttaria darwinii* was shown here to have a SSU sequence near that of *Chalara austriaca*, *Ch. microchona*, *Spathularia flavida* and *Bulgaria inquinans*, supporting placement of *Cyttaria* in the *Leotiales*. The anamorphic fungus *Phialophora gregata* was found to have a sequence similar to that of the *Leotiales*, thus supporting Gams' contention (this volume) that *Phialophora* is polyphyletic.

The grouping of *Byssoascus striatisporus* (*Onygenales*, *Myxotrichaceae*) within the *Leotiales* based on our SSU and LSU analyses is surprising because it is a cleistothecial genus (Alexopoulos *et al.*, 1996). Similarly, SSU analysis also placed *Myxotrichum* and *Pseudogymnoascus* (*Onygenales*, *Myxotrichaceae*) in the *Leotiales* (data not shown). Sugiyama *et al.* (1999) suggested that the *Myxotrichaceae* are distinct from the other *Onygenales* and have some affinity to the *Leotiales*. Similarly, our analyses of the SSU and LSU sequences of *Erysiphales* (data not shown) place these

genera in the *Leotiales*, consistent with the SSU analyses of Sugiyama *et al.* (1999).

The data were clear in the inference that *Ceratocystis* and six *Chalara* species form a monophyletic group, but the taxonomic placement of the genus *Ceratocystis* is not clear from SSU and LSU data. Consistent with earlier studies (Hausner *et al.*, 1993; Spatafora & Blackwell, 1994; Rehner & Samuels, 1995), our data suggest an affinity of *Ceratocystis* with the *Phyllachorales* and the *Microascales*. *Ceratocystis* has been considered to be in its own family within the *Microascales* (Alexopoulus *et al.*, 1996), an order that contains genera with perithecia and sticky ascospore masses suitable for dispersal by insects. However, *Ceratocystis* species differ from species classified in the *Microascales* by their pathogenicity to plants.

Among the 23 *Chalara* species studied, six species are reported to be plant pathogens, which is characteristic of the genus *Ceratocystis* (Kile, 1993). Two of the six species, *Ch. australis* and *Ch. neocaledoniae*, are capable of forming perithecia with inviable ascospores when paired with *MAT*-1 strains of *Ceratocystis eucalypti* or *C. virescens*, respectively (Harrington *et al.*, 1998). All available strains of these two *Chalara* species are *MAT*-2, but it is possible that *MAT*-1 strains exist in nature. *Chalara australis*, *Ch. neocaledoniae*, *C. eucalypti*, and *C. virescens* have nearly identical SSU and LSU sequences, have similar anamorphs, and have a similar biology (Harrington *et al.*, 1998). Given the capacity to produce sexual fruiting bodies in interspecific pairings, it seems likely that *Ch. australis* and *Ch. neocaledoniae* will eventually be shown to be sexual species.

Four other *Chalara* species in the *Ceratocystis* clade, however, are not known to produce perithecia and may represent truly asexual species. *Chalara elegans* is well known as a soilborne pathogen of many genera of plants around the world, while *Ch. populi*, *Ch. ovoidea*, and *Ch. thielavioides* are less known plant pathogens (Kiffer & Delon, 1983; Kile, 1993). In addition to the *Chalara* anamorph, each of these four species form thick-walled, pigmented chlamydospores at the tips of specialized conidiophores, and the term aleurioconidia has been used to describe these conidia. Aleurioconidia are also found in *C. fimbriata*, *C. paradoxa*, and *C. adiposa*, and the LSU and SSU sequences of the four *Chalara* species were very similar to those of the *Ceratocystis* species with aleurioconidia.

The generic concept of *Thielaviopsis* Went, typified by *T. paradoxa* (de Seynes) Höhnel, the anamorph of *C. paradoxa*, is based on the presence of both *Chalara* and aleurioconidial synanamorphs (Nag Raj & Kendrick, 1975). The name *Thielaviopsis* is available for *Chalara* species with *Ceratocystis* affinities. *Chalara elegans* is better known as *T. basicola* (Berk. & Br.) Ferr. and, based on our sequence analyses, we prefer the latter name. The four aleurioconidia-forming *Chalara* species have very similar rDNA sequences and seem to have a similar biology (soilborne root pathogens), and it is possible that they represent an asexual lineage within *Ceratocystis*.

Based on the numerous genera and orders with reported chalara-like anamorphs, we were somewhat surprised that the *Chalara* species we studied fell into only three groups: the *Leotiales*, *Ceratocystis*, and *Ch. hyalina*. If cultures of more *Chalara* species had been available, we may have found that their rDNA sequences would have placed them in the *Sordariales* (with *Cryptendoxyla*), with species of *Pyxidiophora* (*Laboulbeniales*) or with species of the *Trichosphaeriales* with chalara-like anamorphs.

Unfortunately, no culture of the type species of *Chalara*, *Ch. fusidioides*, was available, but the description and biology of this species, especially its slow growth rate on agar media (Nag Raj and Kendrick, 1975), suggest that it may have Leotialean affinities. If *Chalara* is to represent species with Leotialean affinities, then *Ch. hyalina* needs to be placed elsewhere. The six plant-pathogenic *Chalara* species with *Ceratocystis* affinities should also be excluded from *Chalara*, and the generic name *Thielaviopsis* is recommended.

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Table 4. Sequences of additional taxa included in the analyses

Classification[a]	Species	GenBank[b] SSU rDNA	GenBank[b] LSU rDNA
Chaetothyriales	*Capronia fungicola* (Samuels & E. Müller) E. Müller *et al.*	L35298*	AF050246*
anamorphic *Chaetothyriales*	*Hortaea werneckii* (Horta) Nishimura & Miyaji	Y18693*	–
Cyttariales	*Cyttaria darwinii* Berk.	U53369	–
Dothideales	*Mycosphaerella mycopappi* Funk & Dorworth	U43449*	U43480*
anamorphic *Dothideales*	*Aureobasidium pullulans* (de Bary) G. Arnaud	–	AF050239*
Erysiphales	*Phyllactinia guttata* (Wallr. : Fr.) Lév.	AF021796*	–
Eurotiales and other cleistothecial ascomycetes	*Albertiniella polyporicola* (Jaczewski) Malloch & Cain	–	AF096185*
	Cephalotheca sulfurea Fuckel	–	AF096188*
	Connersia rilstonii (C. Booth) Malloch	–	AF096189*
	Eremascus fertilis Eidam	–	U94940*
	Monascus purpureus Went	M83260*	–
	Pleuroascus nicholsonii Massee & E.S. Salmon	–	AF096196*
	Pseudeurotium zonatum van Beyma	–	AF096198*
anamorphic *Eurotiales*	*Oosporidium margaritiferum* Stauz	–	U40090*
	Paecilomyces variotii Bainier	Y13996*	–
Halosphaeriales	*Halosphaeriopsis mediosetigera* (Cribb & J.W. Cribb) T.W. Johnson	–	U46887*
Hypocreales	*Gibberella pulicaris* (Fr. : Fr.) Sacc.	–	AF006326*
	Hypocrea lutea (Fr.) Sacc.	D14407	U00739
	Hypomyces chrysospermus Peck	M89993	–
	Nectria zonata Seaver	–	U17424*
anamorphic *Hypocreales*	*Clonostachys rosea* (Link : Fr.) Schroers	–	U00736*
	Geosmithia lavendula J. Pitt	D14405	AF033385*
	Paecilomyces tenuipes Bainier	D85136	–
	Verticillium lecanii (A. Zimmerman) Viégas	–	U17421*
Leotiales	*Botryotinia fuckeliana* (de Bary) Whetzel	AL114939	–
	Bulgaria inquinans (Pers. : Fr.) Fr.	AJ224362	–
	Leotia lubrica Pers. : Fr.	L37536	–
	Monilinia fructicola (G. Wint.) Honey	AF010505	–
	Sclerotinia sclerotiorum (Lib.) de Bary	X69850	–
	Sclerotinia veratri Cash and R. W. Davidson	–	AF113739
	Spathularia flavida (Fr. : Fr)	Z30239	AF113735

Microascales	*Microascus cirrhosus* Zukal	M89994	–
	Microascus trigonosporus C.W. Emmons & B.O. Dodge	–	U47835
	Petriella setifera (J.C. Schimdt) Curzi	U43908	AF043596
	Pseudallescheria boydii (Shear) McGinnis,Padhye & Ajello	M89782	–
Onygenales	*Myxotrichum deflexum* Berk. (*Myxotrichaceae*)	AB015776*	–
	Onygena equina (Willd.) Pers. (*Onygenaceae*)	U45442*	–
	Pseudogymnoascus roseus Raillo (*Myxotrichaceae*)	AB015777*	–
Ophiostomatales	*Ophiostoma piliferum* (Fr. : Fr.) Syd. & P. Syd.	–	U47837*
anamorphic *Phyllachorales*	*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.	M55640	–
	Colletotrichum truncatum (Schweinitz) Andrus & Moore	–	Z18978
	Verticillium dahliae Kleb.	U33637	–
Pleosporales	*Cucurbitaria elongata* (Fr. : Fr.) Grev.	U42482*	–
	Herpotrichia juniperi (Duby) Petr.	U42483*	–
	Leptosphaeria doliolum (Pers. : Fr.) Ces. & De Not.	U43457*	U43475*
	Ophiobolus herpotrichus (Fr. : Fr.) J.C. Walker	–	OHU43471*
	Pleospora betae (Berl.) Nevodovsky	U43466*	U43483*
Saccharomycetales	*Saccharomyces cerevisae* Meyen ex Hansen	Z75578*	U44806
Sordariales	*Chaetomium elatum* Kunze & Schmidt : Fr.	M83257	–
	Chaetomium globosum Kunze : Fr.	–	U47825*
	Neurospora crassa Shear & B. Dodge	X04971	M38154
	Sordaria fimicola (Roberge ex Desm.) Ces. & De Not.	X69851	–
anamorphic *Sordariales*	*Kionochaeta ivoriensis* (Rambelli & Lunghini) P.M. Kirk & B. Sutton	AB003787*	–
Xylariales	*Xylaria hypoxylon* (L. : Fr.) Grev.	–	U47841*
	Xylaria curta Fr.	–	U47841*
Basidiomycetes	*Bulleromyces albus* Boekhout & Fonseca	X60179	AF075500

[a] Hawksworth *et al.* (1995) was the primary source used for classification of species within orders.

[b] * Sequences used for comparison but not included in phylogenetic trees.

Table 5. Primers used for the amplification and sequencing of rDNA

Primer	Sequence (5'–3')	Source
LROR	ACCCGCTGAACTTAAGC	Vilgalys & Hester, 1990
LR5	TCCTGAGGGAAACTTCG	Vilgalys & Hester, 1990
LR3	CCGTGTTTCAAGACGGG	Vilgalys & Hester, 1990
SR9R	QAGAGGTGAAATTCT	Elwood *et al.*, 1985
SR10R	TTTGACTCAACACGGG	Elwood *et al.*, 1985
NS8	TCCGCAGGTTCACCTACGGA	White *et al.*, 1990
ITS2	GCTGCGTTCTTCATCGATGC	White *et al.*, 1990