

Cystofilobasidiales, a new order of basidiomycetous yeasts

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The order Cystofilobasidiales is described for teleomorphic basidiomycetous yeasts with holobasidia and teliospores. Their septa have dolipores, but lack parenthesomes. D-Glucuronate, nitrate and nitrite are assimilated and myo-inositol is usually assimilated. Coenzyme Q has 8 or 10 isoprenologues. 25S and 18S rDNA sequence analysis indicates a monophyletic branch within the Tremellomycetidae of the Hymenomycetes. Cystofilobasidium is the type genus.

Keywords: phylogeny, basidiomycetes, yeast, rDNA, Cystofilobasidiales

INTRODUCTION

The assessment of relationships among basidiomycetous yeasts has been hampered by the absence of phylogenetically informative characters; affinity among these organisms was inferred using morphological, biochemical and physiological characters (von Arx *et al.*, 1977; Boekhout *et al.*, 1993; van der Walt, 1987; Kurtzman & Fell, 1998). Features considered useful were cell wall composition (Prillinger *et al.*, 1993; Roeijmans *et al.*, 1998; Sugiyama *et al.*, 1985; Weijman & Golubev, 1987; Weijman & Rodrigues de Miranda, 1988), septal ultrastructure (Boekhout *et al.*, 1992; McLaughlin *et al.*, 1995; Moore, 1987; Suh *et al.*, 1993), morphology of the basidium (Bandoni, 1995; Boekhout *et al.*, 1993; Oberwinkler, 1987), killer sensitivity patterns (Golubev & Boekhout, 1995) and specific physiological traits such as assimilation of *myo*-inositol and D-glucuronate (Golubev, 1989), production of extracellular starch-like compounds (Boekhout *et al.*, 1993; Sampaio & Fonseca, 1995), assimilation of nitrate and nitrite, vitamin requirements (Sampaio & Fonseca, 1995) and ubiquinone composition (Sugiyama *et al.*, 1985; Yamada *et al.*, 1987). As a result, both anamorphic and teleomorphic basidiomycetous yeasts were divided into two or three main groups (Boekhout *et al.*, 1993; Sampaio & Fonseca, 1995; van der Walt, 1987).

Recent molecular phylogenetic studies of basidiomycetous yeasts, using 18S rDNA sequences, indicated

the presence of three classes of Basidiomycota, namely Hymenomycetes, Urediniomycetes and Ustilaginomycetes (Swann & Taylor, 1995a, b). Based on studies of 25S rDNA sequences, yeast stages were found to occur in all of these classes (Boekhout *et al.*, 1995; Fell *et al.*, 1995). A monophyletic cluster of species occurs within the Tremellomycetidae of the Hymenomycetes (Swann & Taylor, 1995c), that we propose to recognize as the new order Cystofilobasidiales.

METHODS

Strains. Species and strains investigated are shown in Table 1. Wherever possible, type strains were investigated. Many filamentous species, such as species of *Tremella* and *Sirobasidium*, do not have designated living type strains.

Carbohydrate patterns and coenzyme Q measurement. Carbohydrate patterns were determined by analysis of whole-cell hydrolysates with GLC/MS (Roeijmans *et al.*, 1998). Coenzyme Q (CoQ) was extracted with hexane after saponification of intact cells and purification by TLC. Coenzyme Q systems were identified by reverse-phase TLC (Yamada & Kondo, 1973; Hiraishi *et al.*, 1984) and by MS. In the latter technique, 3 µl crude extract was inserted in the quartz sample tube of the direct inlet probe and probe temperature was programmed from 50 to 400 °C. The CoQ components evaporated when the probe temperature exceeded 300 °C. Mass spectra were recorded with an ionizing energy of 70 eV (electron impact) and the CoQ systems were identified by their molecular mass.

Sequencing and sequence analysis. Forward and reverse strands of the ~650 bp region at the 5' end of the 18S rDNA were sequenced with an Li-Cor Automated Sequencer using

The GenBank accession numbers for the sequences reported in this paper are AF075463–AF075526.

Table 1. List of strains examined

Species	CBS strain no.*	GenBank accession no.
<i>Bullera crocea</i> Buhagiar	6714 ^T	AF075508
<i>Bullera globispora</i> Johri & Bandoni	6981 ^T	AF075509
<i>Bullera oryzae</i> Nakase & Suzuki	7194 ^T	AF075511
<i>Bullera pseudoalba</i> Nakase & Suzuki	7227 ^T	AF075504
<i>Bullera unica</i> Hamamoto & Nakase	8290 ^T	AF075524
<i>Bulleromyces albus</i> Boekhout & Fonseca	501	AF075500
<i>Cryptococcus aerius</i> (Saito) Skinner	155 ^T	AF075486
<i>Cryptococcus albidus</i> (Saito) Skinner	142 ^T	AF075474
<i>Cryptococcus antarcticus</i> Vishniac & Kurtzman	7687 ^T	AF075488
<i>Cryptococcus aquaticus</i> (Jones & Slooff) Rodrigues de Miranda & Weijman	5443 ^T	AF075470
<i>Cryptococcus cellulolyticus</i> Nakase <i>et al.</i>	8294 ^T	AF075525
<i>Cryptococcus diffluens</i> Zach (Lodder & Kreger-van Rij)	160 ^T	AF075502
<i>Cryptococcus dimennae</i> Fell & Phaff	5770 ^T	AF075489
<i>Cryptococcus feraegula</i> Saéz & Rodrigues de Miranda	7201	AF075487
<i>Cryptococcus flavus</i> (Saito) Phaff & Fell	331 ^T	AF075497
<i>Cryptococcus friedmannii</i> Vishniac	7160 ^T	AF075478
<i>Cryptococcus fuscescens</i> Golubev	7189 ^T	AF075472
<i>Cryptococcus heveanensis</i> (Groenewege) Baptist & Kurtzman	569 ^T	AF075467
<i>Cryptococcus hungaricus</i> (Zsolt) Phaff & Fell	4214 ^T	AF075503
<i>Cryptococcus laurentii</i> (Kufferath) Skinner	139 ^T	AF075469
<i>Cryptococcus luteolus</i> Skinner	943 ^T	AF075482
<i>Cryptococcus macerans</i> (Frederiksen) Phaff & Fell	2425 ^{MT}	AF075477
<i>Cryptococcus podzolicus</i> (Babjeva & Reshetova) Golubev	6819 ^T	AF075481
<i>Cryptococcus skinneri</i> Phaff & do Carmo-Sousa	7890 ^T	AF075494
<i>Cryptococcus terreus</i> di Menna	1895 ^T	AF075479
<i>Cryptococcus vishniaci</i> Vishniac & Hempfling	7110 ^T	AF075473
<i>Cystofilobasidium bisporidii</i> (Fell <i>et al.</i>) Oberwinkler & Bandoni	6347 ^{MT}	AF075464
<i>Cystofilobasidium capitatum</i> (Fell <i>et al.</i>) Oberwinkler & Bandoni	6358 ^T	AF075465
<i>Cystofilobasidium infirmominiatum</i> (Fell <i>et al.</i>) Hamamoto <i>et al.</i>	323 ^T	AF075505
<i>Cystofilobasidium lari-marini</i> (Saéz & Nguyen) Fell & Statzell-Tallman	7420 ^T	AF075466
<i>Fellomyces fuzhouensis</i> (Yue) Yamada & Banno	6133	AF075506
<i>Filobasidiella neoformans</i> var. <i>bacillispora</i> Kwon-Chung	6289 ^{MT}	AF075526
<i>Filobasidiella neoformans</i> var. <i>neoformans</i> Kwon-Chung	132 ^T	AF075484
<i>Filobasidium capsuligenum</i> Rodrigues de Miranda	4736 ^T	AF075501
<i>Filobasidium floriforme</i> Olive	6241 ^T	AF075498
<i>Filobasidium globisporum</i> Bandoni & Oberwinkler	7642	AF075495
<i>Filobasidium uniguttulatum</i> Kwon-Chung	1730 ^T	AF075468
<i>Kockvoella thailandica</i> Nakase <i>et al.</i>	7552 ^T	AF075516
<i>Mrakia frigida</i> (Fell <i>et al.</i>) Yamada & Komagata	5270 ^T	AF075463
<i>Rhodotorula diffluens</i> (Ruijten) von Arx & Weijman	5233 ^T	AF075485
<i>Rhodotorula foliorum</i> (Ruijten) Rodrigues de Miranda & Weijman	6370	AF075499
<i>Rhodotorula philyla</i> (van der Walt <i>et al.</i>) Rodrigues de Miranda & Weijman	6272 ^T	AF075471
<i>Sirobasidium intermedium</i> Kundalkar & Patil (yeast phase)	7805	AF075492
<i>Sirobasidium magnum</i> Boedijn	6803	AF075475
<i>Sporobolomyces falcatus</i> Santa Maria	7368 ^T	AF075490
<i>Sterigmatosporidium polymorphum</i> Kraepelin & Schulze	8088 ^T	AF075480
<i>Tremella fuciformis</i> Berkeley (yeast state)	6970	AF075476
<i>Tremella mesenterica</i> Retzius: Fries (yeast state)	6973	AF075518
<i>Tremella moriformis</i> Smith & Sowerby (yeast state)	7810 ^T	AF075493
<i>Trichosporon aquatile</i> Hedrick & Dupont	5973 ^T	AF075520
<i>Trichosporon asteroides</i> (Rischin) Ota	2481 ^T	AF075513
<i>Trichosporon brassicace</i> Nakase	6382 ^T	AF075521
<i>Trichosporon cutaneum</i> (de Beurmann <i>et al.</i>) Ota	2466 ^T	AF075483
<i>Trichosporon domesticum</i> Sugita <i>et al.</i>	8280 ^T	AF075512
<i>Trichosporon dulcitum</i> (Berhout) Weijman	8257 ^T	AF075517
<i>Trichosporon laibachii</i> (Windisch) Guého & Smith	5790 ^T	AF075514
<i>Trichosporon loubieri</i> (Morenz) Weijman	7065 ^T	AF075522
<i>Trichosporon mucoides</i> Guého & Smith	7625 ^T	AF075515
<i>Trichosporon ovoides</i> Behrend	7556 ^T	AF075523
<i>Trimorphomyces papilionaceus</i> Oberwinkler & Bandoni	445.92	AF075491
<i>Udeniomycetes megalosporus</i> (Nakase & Suzuki) Nakase & Takematsu	7236 ^T	AF075510
<i>Udeniomycetes puniceus</i> (Komagata & Nakase) Nakase & Takematsu	5689 ^T	AF075519
<i>Udeniomycetes pyricola</i> (Stadelmann) Nakase & Takematsu	6754 ^T	AF075507
<i>Xanthophyllomyces dendrorhous</i> Golubev	7918 ^T	AF075496

* CBS, Centraalbureau voor Schimmelcultures, The Netherlands. ^T, Type strain; ^{MT}, mating type.

† Note added in proof. CBS 7890 was originally identified as *Cryptococcus skinneri* in the CBS database. Subsequent examination of CBS 5029^T, the type strain of *C. skinneri*, demonstrated that the two strains are phylogenetically distinct. CBS 5029^T occurs in the same clade as *C. luteolus* in Fig. 1. CBS 7890 represents an undescribed species.

a standard protocol with forward primer 5' GCATATCA-ATAAGCGGAGGAAAAG and reverse primer 5' GGTC-CGTGTTCAAGACG. Alignments were prepared with MegAlign (DNAStar) and visually corrected. Phylogenetic trees were computed with PAUP (test version 4.0d61, used with the permission of D. Swofford) using parsimony analysis (heuristic search, random stepwise addition, nearest-neighbour interchange, 100 maximum trees). Bootstrap analysis was based on 1000 replicates.

RESULTS AND DISCUSSION

Yeasts classified in the Tremellomycetidae (Hymenomycetes) are characterized by the presence of dolipore septa, xylose in the cell walls and, with some exceptions, utilization of myo-inositol and D-glucuronate, and production of starch-like compounds. Fig. 1 is a representative analysis of the Tremellomycetidae; the number of species in this figure was limited to increase the visual clarity of the tree. There are two major branches, one of which contains genera of the Tremellales/Filobasidiales and the other branch consists of the teleomorphic genera *Mrakia* and *Cystofilobasidium*, the anamorphic genus *Udenomyces* and two species of *Cryptococcus*. The Tremellales and Filobasidiales are characterized by a sexual cycle that lacks a teliosporic probasidium and by the morphology of the septal pores. Species of *Bulleromyces*, *Sirobasidium*, *Sterigmatosporidium*, *Tremella* and relatives, and *Filobasidium* have Tremellales-type dolipoles with tubular, vesiculate or 'U'-shaped parenthesomes (Boekhout *et al.*, 1993; Kwon-Chung *et al.*, 1995; McLaughlin *et al.*, 1995; Moore & Kreger-van Rij, 1972; Oberwinkler & Bandoni, 1982). The reported absence of a parenthosome in *Filobasidium floriforme* can be questioned, as a Tremellales-type parenthosome appears to be present in Fig. 4 of Moore & Kreger-van Rij (1972). Parenthesomes are generally considered as modified compartments of the endoplasmic reticulum (Moore, 1985), which may play an important role in intercellular signalling and maintenance of homeostasis (Mueller *et al.*, 1998).

There are two sub-branches within the Tremellales/Filobasidiales: one sub-branch, which is weakly supported (58 %) by bootstrap analysis, consists of species of *Tremella*, *Bulleromyces*, *Filobasidiella*, etc. The anamorphic genus *Trichosporon*, which is included in this cluster, is supported (bootstrap value 81 %) as a cohesive group. This genus, which is characterized by the presence of arthroconidia, was studied in detail by Guého *et al.* (1992). The other sub-branch, which has a bootstrap support of 90 %, includes *Filobasidium* and several species of *Cryptococcus*. These data suggest that the Filobasidiales are phylogenetically distinct from the Tremellales. The sequence analysis also demonstrates a separate Cystofilobasidiales clade within the Tremellomycetidae of the Hymenomycetes, with a bootstrap value of 87 % (Fig. 1). Two subclades

can be discerned: (1) *Mrakia frigida*, *Cryptococcus aquaticus* and *Udenomyces* spp. (90 % support); and (2) *Cryptococcus feraegula*, *Cryptococcus macerans* and *Cystofilobasidium* spp. (100 %). These results agree with 18S rDNA data, which support the *Cystofilobasidium*-*Mrakia* clade with 98 % bootstrap values (Suh & Nakase, 1995; Swann & Taylor, 1995a, b). The two teleomorphic genera in these clades, *Mrakia* and *Cystofilobasidium*, produce teliospores, a characteristic not found among other hymenomycetous yeasts. Metabasidia differ between the two genera: teliospore germination in *Mrakia* results in short ($\sim 8 \times 5 \mu\text{m}$) holo- or phragmometabasidia; whereas, most species of *Cystofilobasidium* produce a long, thin (up to $80 \times 2.7 \mu\text{m}$) holobasidium with a capitate apex (Kwon-Chung, 1998).

The distribution of other character states among the species of Cystofilobasidiales is given in Table 2. Two different CoQ systems occur: CoQ 8 occurs in all species except for *Udenomyces* spp., which contain CoQ 10 (Boekhout *et al.*, 1992; Guého *et al.*, 1993; Nakase & Suzuki, 1986; Nakase & Takematsu, 1992; Suh & Sugiyama, 1993; Sugiyama *et al.*, 1985; Yamada & Komagata, 1987; Yamada & Kondo, 1972, 1973; Yamada & Matsumoto, 1989). The different genes involved in the biosynthesis of distinct CoQ molecules (CoQ 7, 8, 9 or 10) may be present in a single organism. This possibility is suggested by the report of the occurrence of minor CoQ fractions in concert with one or two major CoQ fractions (Billon-Grand, 1988; Kuraishi *et al.*, 1985) and by the observation that the relative proportions of these different CoQ fractions are influenced by environmental conditions (Billon-Grand, 1989). If this supposition is true, the presence of two different major CoQ molecules among the Cystofilobasidiales may be the result of differences in gene expression with limited phylogenetic significance. It should be noted, however, that CoQ 10 is confined to the ballistoconidia-forming species and CoQ 8 to the teliospore-forming yeasts and closely related anamorphs.

All species of Cystofilobasidiales contain xylose in their cell walls (von Arx & Weijman, 1979; Boekhout *et al.*, 1992; Elinov *et al.*, 1974; Golubev, 1995; Gorin & Spencer, 1970; Roeijmans *et al.*, 1989; Sugiyama *et al.*, 1985; Suh & Sugiyama, 1993; Weijman, 1979; Weijman & Rodrigues de Miranda, 1988). D-Glucuronate, nitrate and nitrite are assimilated by all species, myo-inositol is used by most species, except *Cryptococcus aquaticus*, most species are dependent on thiamine and biotin, and fermentative capabilities occur in *Cystofilobasidium lari-marinii* (Barnett *et al.*, 1990; Kwon-Chung, 1998). Hyphal septa of investigated species of the Cystofilobasidiales, namely *Mrakia frigida* and *Cystofilobasidium* spp., have dolipoles without parenthesomes (Oberwinkler *et al.*, 1983; Suh & Sugiyama, 1993; Suh *et al.*, 1993). The argument can be presented that dolipoles without parenthesomes are plesiomorphic when compared with dolipoles with vesiculate, tubular or 'U'-shaped,

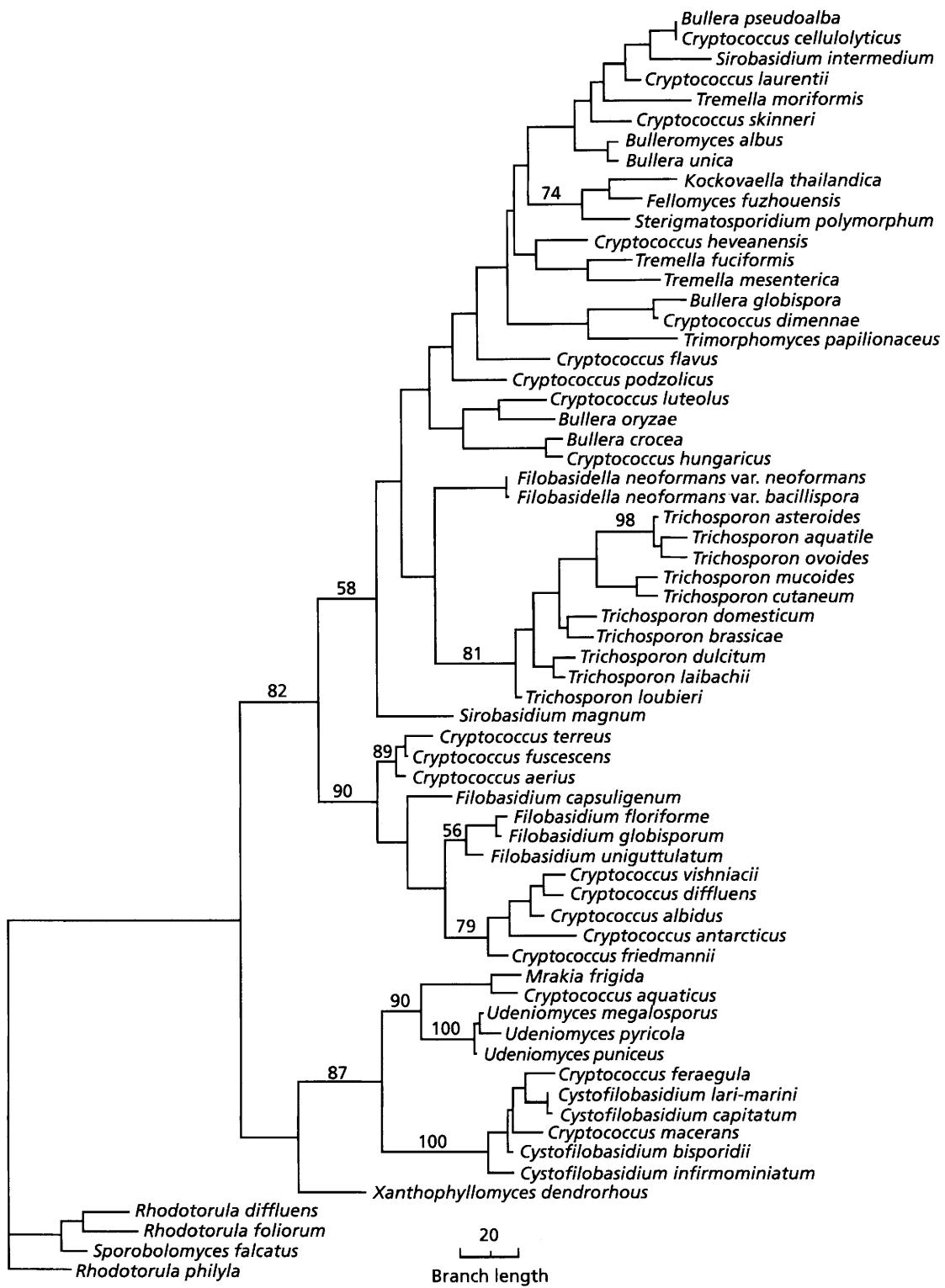


Fig. 1. Phylogenetic tree of yeasts of the Tremellomycetidae derived from parsimony analysis (heuristic search, random stepwise addition, nearest-neighbour interchange, 100 maximum trees). Branch lengths are proportional to the number of nucleotide differences. The numbers given on branches are the frequencies (percentages) that a given branch appeared in 1000 bootstrap replicates (PAUP test version 4.0d61, used with the permission of D. Swofford). Species of Urediniomycetes (*Rhodotorula* and *Sporobolomyces*) were used as an outgroup.

Table 2. Summary of salient characteristics of Cystofilobasidiales species

CoQ, Coenzyme Q; Xylose, presence of xylose in cell-free extract; Telio, formation of teliospore; Basidia, absence of basidium (–) or presence of holometabasidium (HOLO); Septa, septal type unknown (?) or presence of dolipore (DOLI); Inositol, assimilation of *myo*-inositol; D-Gluc, assimilation of D-glucuronate; Starch, production of starch-like compounds; NO₃ and NO₂, assimilation of nitrate and nitrite; w/o Thiamine/Biotin, growth in the absence of either of those vitamin sources. v, Variable reaction; w, weak reaction, D, delayed; *, new data. Other data from Barnett *et al.* (1990) and Kurtzman & Fell (1998).

Species	CoQ	Xylose	Telio	Basidia	Septa	Inositol	D-Gluc	Starch	NO ₃	NO ₂	w/o Thiamine	w/o Biotin
<i>Cryptococcus aquaticus</i>	8*	+	–	–	?	–	+	+	+	+	–	–
<i>Cryptococcus feraeagula</i>	8*	+ *	–	–	?	+	+	+	+	+	–	–
<i>Cryptococcus macerans</i>	8*	+	–	–	?	+	+	+	+	+	–	–
<i>Cystofilobasidium bisporidii</i>	8	+	+	HOLO	DOLI	+	+	v	+	+	–	–
<i>Cystofilobasidium capitatum</i>	8	+	+	HOLO	DOLI	+	+	+	+	+	–	v
<i>Cystofilobasidium infirmominiatum</i>	8	+	+	HOLO	DOLI	+	+	+	+	+	–	–
<i>Cystofilobasidium lari-marini</i>	8	+	+	HOLO	DOLI	v	+	+	+	+	–	,D
<i>Mrakia frigida</i>	8	+	+	HOLO	DOLI	v	+	+	+	+	–	,D
<i>Udenomyces megalosporus</i>	10	+	–	–	?	–	+	+	+	+	–	–
<i>Udenomyces pyricola</i>	10	+	–	–	?	+	+	w	+	+	–	–
<i>Udenomyces puniceus</i>	10	+	–	–	?	+	+	+	+	+	–	–

plate-like or perforate parenthesomes. However, detailed studies on the biogenesis, differentiation and function of the septum–parenthesome complex in different groups of hymenomycetous fungi, e.g. Auriculariales, Cystofilobasidiales, Filobasidiales, Exidiales, Tremellales and higher fungi (e.g. Agaricales), are necessary to understand the evolutionary importance of these structures. Preparation techniques, such as high-pressure freezing, freeze substitution and cryo-electron microscopy, are essential to avoid artifacts (Mueller *et al.*, 1995; Orlovich & Ashford, 1994) which may hamper correct interpretations. This requirement is illustrated by conflicting results in different studies on the septal ultrastructure of *Filobasidiella depauperata* (Kwon-Chung *et al.*, 1995; Rhodes *et al.*, 1981).

Our analysis of rDNA sequences demonstrates that three species of *Cryptococcus* are members of the Cystofilobasidiales (Fig. 1). Based on this apparent relationship, the hypothesis can be offered that *Cryptococcus macerans* and *Cryptococcus feraeagula* may have a *Cystofilobasidium*-type life cycle, whereas the life cycle of *Cryptococcus aquaticus* may be similar to *Mrakia*. *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) may be related to Cystofilobasidiales as indicated by 18S data (Swann & Taylor, 1995c); however, inclusion in this clade is not supported by bootstrap analysis (Fig. 1) using 25S rDNA data. Parsimony analysis indicated that two other species, *Trichosporon pullulans* and *Itersonila perplexans*, may be weakly related to the Cystofilobasidiales (Fell *et al.*, 1995), but bootstrap analysis did not support their inclusion. Both species differ from the Cystofilobasidiales by the presence of CoQ 9. As more data accumulates regarding peripherally related species, our vision of Cystofilobasidiales may alter. Similarly, the present definitions of the Tremellales and Filobasidiales must be evaluated to consider such problems as the phylogenetic placement of *Filobasidiella*.

Latin diagnosis of Cystofilobasidiales Boekhout & Fell

Cystofilobasidiales, order novus Basidiomycetum, species plerumque zymosae; taxa meiosporica, heterothallica vel ipsifertilia. Teliosporae vulgo formatae; basidia si praesentia, unicellularia. Coenzymum Q 8 vel 10. Parietes cellularum xylosium continentis. Septa hypharum plerumque doliporis parenthesomate parentibus praedita, sed nonnumquam imperforata. D-Glucuronatum, nitratum et nitritum assimilantur. Sequentiae rDNA positionem in hymenomycetibus indicantes. Genus typicum *Cystofilobasidium* Oberwinkler & Bandoni.

Description of Cystofilobasidiales Boekhout & Fell

Basidiomycetous fungi, usually with a free-living yeast phase. Meiotic taxa are heterothallic or self-sporellating (secondarily homothallic?). Teliospores are present. Basidia, if present, are unicellular. CoQ 8 or 10 is present. Cell walls contain xylose. Hyphal septa usually have dolipores without a parenthesome. D-Glucuronate, nitrate and nitrite are assimilated and *myo*-inositol is usually assimilated. 25S rDNA sequences form a monophyletic branch within the Tremellomycetidae. Type genus is *Cystofilobasidium* Oberwinkler & Bandoni.

The following meiotic taxa are included in the Cystofilobasidiales: *Cystofilobasidium*, including *Cystofilobasidium bisporidii* (Fell *et al.*) Oberwinkler & Bandoni, *Cystofilobasidium capitatum* (Fell *et al.*) Oberwinkler & Bandoni, *Cystofilobasidium infirmominiatum* (Fell *et al.*) Hamamoto *et al.*, *Cystofilobasidium lari-marini* (Saëz & Nguyen) Fell & Statzell-Tallman; *Mrakia*, including *Mrakia frigida* (Fell *et al.*) Yamada & Komagata. The following taxa are only known in their mitotic state, but 25S rDNA sequences indicate that they belong to this order: *Cryptococcus*, including *Cryptococcus aquaticus* (Jones

& Slooff) Rodrigues de Miranda & Weijman (*Mrukia* branch), *Cryptococcus feraegula* Saéz & Rodrigues de Miranda, *Cryptococcus macerans* (Frederiksen) Phaff & Fell (*Cystofilobasidium* branch); *Udeniomyces*, including *Udeniomyces megalosporus* (Nakase & Suzuki) Nakase & Takematsu, *Udeniomyces puniceus* (Komagata & Nakase) Nakase & Takematsu, *Udeniomyces pyricola* (Stadelmann) Nakase & Takematsu (*Udeniomyces* branch).

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