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Phylogenetic relationships of *Cryptococcus neoformans* and some related basidiomycetous yeasts determined from partial large subunit rRNA sequences

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Abstract

The genus *Cryptococcus* was found to be heterogeneous on the basis of partial rRNA sequences. The human-pathogenic species *C. neoformans*, comprising 4 serotypes and having *Filobasidiella neoformans* and *F. bacillispora* as teleomorphs, was found at a relatively large distance from *Filobasidium*. Serotypes B and C had identical sequences, while in A and D they were different, with D closer to B and C than to A. *Filobasidiella depauperata*, which lacks a yeast-like anamorph, clustered with *F. neoformans*.

The genus *Filobasidium* was clearly separated from *Filobasidiella* and clustered with *C. albidus*, *C. kuetzingii*, *C. gastricus*, *C. lupi*, *C. vishniaciae*, *C. bhutanensis*, *C. aerius*, *C. terreus* and *C. ater*. The latter may represent the anamorph of *Filobasidium elegans*. The orange to red species of *Cryptococcus*, as well as *C. aquaticus* and *C. yarrowii*, were found completely unrelated with these taxa, *C. macerans* being affiliated to *Cystofilobasidium capitatum*.

The genus *Trichosporon* was found relatively homogeneous; it includes *C. humicola*, *C. curvatus* and the filamentous species *Hyalodendron lignicola*. *Cryptococcus flavus* and *C. dimennae* probably belong to the Tremellales, though distances between these species are large. The positions of *C. laurentii* and *C. luteolus* remains to be determined.

Introduction

Cryptococcus Kützing (1833) is a genus of anamorphic basidiomycetous yeasts. Rodrigues de Miranda & Batenburg-van der Vegte (1981) studied the holotype material of the generic type species, *C. mollis* Kützing, with TEM and found it to have multilamellar cell walls. No pure culture of this species had ever been made and the herbarium specimen was difficult to identify with any presently known species. *Cryptococcus neoformans* (Sanfelice) Vuill. was therefore considered to be the representative species of the genus, in accordance with the generic concept accepted at the time. Because of the questionable identity of *C. mollis* and the increasing importance of *C. neoformans* as an opportunistic, AIDS-associated human pathogen, Fell et al. (1989) proposed to conserve *Cryptococcus* with *C. neoformans* CBS 132 as neotype.

For years, *Cryptococcus* was defined as a genus of white yeasts, combining the absence of fermentation with abilities to assimilate inositol as the only

Table 1. Characteristies of the 36 species (56 strains) examined.[1]

Species	Strain N°[2]	Source	CoQ[3]	Serotype[4]
Cystofilobasidium capitatum (Fell et al.) Oberwinkler & Bandoni	CBS 6358^T	zooplancton	Q 8	
Cryptococcus aerius (Saito) Skinner	CBS 155^T	atmosphere	Q10	
C. albidus (Saito) Skinner	CBS 142^T	atmosphere	Q10	
C. aquaticus (Jones & Slooff) Rodrigues de Miranda & Weijman	CBS 5443^T	water scum		
C. ater (Castellani & Cooke) Phaff & Fell	CBS 4685^T	leg ulcer	Q10*	
C. bhutanensis Goto & Sugiyama	CBS 6294^T	soil		
C. curvatus (Diddens & Lodder) Golubev	CBS 570^T	sputum	Q10	
C. "	IP 7167	urine, AIDS		
C. "	IP 3285	CSF, AIDS		
C. dimennae (Fell & Phaff)	CBS 5570^T	pasture	Q 9*	
C. gastricus Reiersöl & di Menna	CBS 1927^T	soil		
C. flavus (Saito) Phaff & Fell	CBS 331^T	atmosphere	Q10	
C. humicola (Daszewska) Golubev	CBS 571^T	soil	Q9	
C. hungaricus (Zsolt) Phaff & Fell	CBS 4214^T	soil	Q10	
C. kuetzingii Fell & Phaff	CBS 1926^T	*Mespilus* sp.		
C. "	CBS 6086	atmosphere		
C. laurentii (Kufferath) Skinner	CBS 139^T	malafu palm wine	Q10	
C. lupi Baharaeen & Vishniac	CBS 6809	grave	Q9	
C. luteolus (Saito) Skinner	CBS 943^T	atmosphere	Q10	
C. macerans (Frederiksen) Phaff & Fell	CBS 2206^T	dew retted flax	Q8	
C. terreus di Menna	CBS 1895^T	soil	Q10	
C. vishniaciae Vishniac & Hempling	CBS 6810^T	soil	Q9	
C. yarrowii Fonseca & van Uden	CBS 7417^T	decaying mushrom	Q10	
Filobasidiella depauperata (Petch) Samson et al.	ATCC 36983^T	dead spider		
F. neoformans Kwon-Chung var. *neoformans* Kwon-Chung	CBS 5756$^{MT\alpha}$	human		A
F. "	CBS 879$^{MT\alpha}$	ulcerated cheek		A
F. "	NIH 68	human		A
F. "	CDC 551	human		A
F. "	CBS 132$^{T,MT\alpha}$	fruit juice	Q10	D
F. "	NIH 52	CSF		D
F. "	NIH 3501$^{MT\alpha}$	bone		D
F. "	NIH 3502^{MTa}	pigeon droppings		D
F. "	ACH Mak.	sputum		D
F. neoformans Kwon-Chung var. *bacillispora* Kwon-Chung	CBS 6956=NIH 444$^{MT\alpha}$	sputum	Q10	B
F. "	CBS 6998=NIH 365^{MTa}	CSF		B
F. "	ACH 32.6M	*Eucalyptus camaldulensis*		B
F. "	ACH Bal2	*E.camaldulensis*		B
F. "	ACH 4725	CSF, AIDS		B
F. "	CBS 6955=NIH 191^{MTa}	CSF		C
F. "	CBS 6993=NIH 18$^{MT\alpha}$	human		C
F. "	CBS 6994=NIH 34$^{MT\alpha}$	CSF		C
F. "	CDC 238	human		C
Filobasidium capsuligenum Rodriguez de Miranda	CBS 4736$^{TMT\alpha}$	wine cellar	Q10	
F. elegans Bandoni & Oberwinkler	CBS 7637^T	*Yucca brevifolia*		
F. floriforme Olive	CBS 6241$^{MT\alpha}$	*Erianthus giganteus*	Q10	
F. globosporum Bandoni & Oberwinkler	CBS 7642^T	*Yucca brevifolia*		
F. uniguttulatum Kwon-Chung	CBS 1730$^{T,MT\alpha}$	infected finger nail	Q9	
Hyalodendron lignicola Diddens	CBS 221.94^T	wood pulp	Q 9*	
Moniliella suaveolens (Lindner) Von Arx	CBS 126.42^T	Milk	Q 9*	
Rhodosporidium toruloides Banno	CBS 315	air	Q9	
Sirobasidium magnum Boedijn	CBS 6804$^{MT\alpha}$	decaying wood	Q 8*	
Sterigmatosporidium polymorphum Kraepelin & Schulze	CBS 8089$^{MT\alpha}$	water logged planks	Q10	
Tremella aurantia Schweinitz	CBS 6965	*Alnus rubra*	Q10	
T. mesenterica Retz	CBS 6973	*Alnus rubra*	Q10	
T. subanomala Coker	CBS 6976	*Salix* sp.	Q10	
Trichosporonoides oedocephalis Haskins & Spencer	CBS 649.66^T	*Apis mellifica*	Q 9*	

carbon source, to hydrolyze urea and to produce polysaccharide capsules and extracellular starch-like material. Based on this broad definition, species of questionable relationship were subsequently added. Phaff & Fell (1970) gave prevalence to inositol assimilation and presence of starch-like compounds over colony colour, and added species previously classified in *Rhodotorula*, viz. *C. infirmominiatus*, *C. hungaricus* and *C. macerans* with red colony colour and *C. flavus* with yellowish colonies. The generic borderlines therefore have to be reestablished on the basis of phylogenetic relationships.

Judging from the presence of dolipores in hyphal septa with more or less developed, vesicular to tubular parenthesomes (Moore & Kreger-van Rij 1972; Kwon-Chung & Popkin 1976; Moore 1978, 1979; Khan et al. 1981; Rhodes et al. 1981), one might suppose that members of *Cryptococcus*, *Filobasidium*, *Filobasidiella*, *Sirobasidium* and *Tremella*, and consequently the orders Filobasidiales and Tremellales, are interrelated. However, many authors have demonstrated a heterogeneity within the genus *Cryptococcus*. Yamada & Kondô (1972, 1973) found three major coenzyme Q systems (Q8, Q9 and Q10) within the genus. Isolates of the species *C. humicola* appeared even to have different co-Q systems (Yamada & Kondô 1972). Golubev & Vagaboda (1977) and Nakase & Komagata (1971) demonstrated marked generic and specific heterogeneity on the basis of mol% G+C of DNA. Yamada et al. (1990) confirmed this heterogeneity by means of 26S rRNA sequencing. Recently, Mitchell et al. (1992), using 5.8S rDNA and ITS sequences, proved that *Filobasidium* and *Filobasidiella* were unrelated.

The present paper aims to show the phylogenetic relationships within the genera *Cryptococcus*, *Filobasidium* and *Filobasidiella*. For comparison, *Sirobasidium*, *Tremella* and *Trichosporon* are included. Special attention has been paid to *Cryptococcus neoformans*, which is known to have four different serotypes. Serotypes A and D are linked to the teleomorph *Filobasidiella neoformans* var. *neoformans*, while serotypes B and C represent *F. neoformans* var. *bacillispora*. In addition, genera of purported systematic affinity were included. Despite a completely different morphology, similarities between *Cryptococcus* and *Trichosporon* were found in septal pore ultrastructure (Guého et al. 1992). In addition, an antigenic resemblance was reported between medical *Trichosporon* species and *Cryptococcus neoformans* (Melcher et al. 1991). Outgroups for cladistic analysis were chosen in heterobasidiomycetous yeasts studied by Guého et al. (1989), supplemented with the filamentous fungi which show possible relationship with Filobasidiales, i.e. *Hyalodendron lignicola, Moniliella suaveolens* and *Trichosporonoides oedocephalis* (De Hoog 1979).

Partial sequences of the most variable domain (D2; Michot et al. 1984) of the large subunit rRNA were applied to establish phylogenetic relationships between the species studied. The two other regions of the large and small subunits previously analyzed by Guého et al. (1989) proved insufficiently variable to distinguish between serotypes of *C. neoformans* and were not retained for this study.

Materials and methods

Cultures

Thirty-six species represented by 56 strains were sequenced. They are listed in Table 1, with indication of reference number, source, ubiquinone system and serotype for the species *Cryptococcus neoformans*. Strains were grown at 28 °C, or at 18 °C in

Legend to Table 1.

[1] The strains of *Trichosporon* spp. added for the phylogenetic comparison are listed in Guého et al., 1992

[2] CBS, Centraalbureau voor Schimmelcultures, Delft and Baarn, The Netherlands' IP, Institut Pasteur, Paris, France' ATCC, American Type Culture Collection, Rockville, USA; CDC, Center for Disease Control, Atlanta, USA; NIH, National Institue for Health, Bethesda, USA; ACH, Adelaide Children's Hospital, North Adelaide, Australia: T, type culture; MT, mating type culture.

[3] Values obtained from the literature: Yamada & Kondo, 1972; Yamada & Kondo, 1973' Baharaeen & Vishnaic, 1982; Vishnaic & Baharaeen, 1982; Yamada et al., 1983; Sugiyama et al., 1985; Sugiyama et al., 1987; Fonseca & van Uden, 1991. Q*, values determined by H.J. Roeymans and W. Zijlstra (CBS, Baarn).

[4] From Ikeda et al., 1982 and Dromer et al., 1992

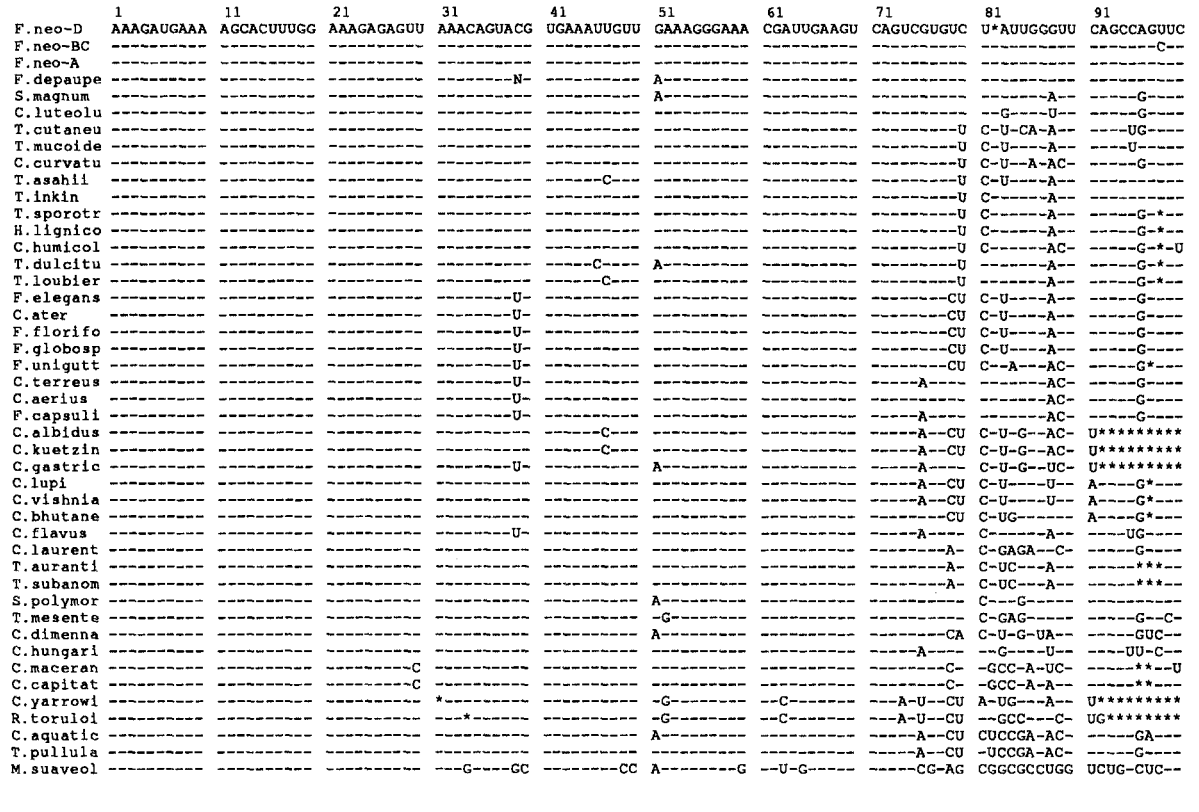

Fig. 1.

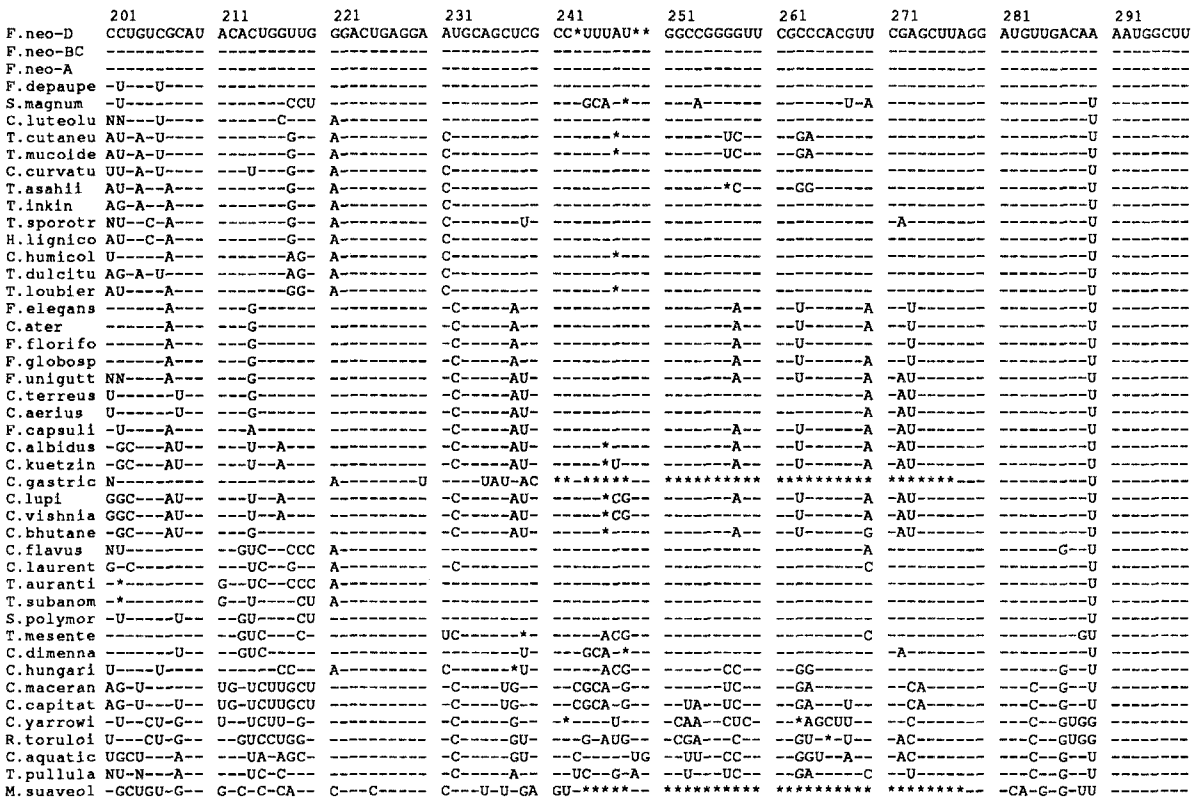

Fig. 1. Alignment of LSU rRNA partial sequences of the D2 variable domain (5' to 3' direction), with *Filobasidiella neoformans* serotype D as model. First base read corresponds to position 626 of *Sacharomyces cerevisiae*. Dashes are used when a particular position is identical to the nucleotide of *F. neoformans*-D, N is used for nucleotides that remain uncertain, * represent deletions necessary for alignment.

case of mesophilic species, for 24 h in 100 ml of shaken liquid medium (1% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract). Cells were harvested by centrifugation, washed, lyophilized and stored at –20 °C until use.

rRNA purification and sequencing

RNA isolation was performed according to Guého et al. (1992). Material was ground in a mortar with sand and liquid nitrogen. The resulting fine powder was transferred to 2 ml-Eppendorf tubes and soaked in 0.9 ml extraction buffer [50 mM hydroxymethylaminomethane (pH 7.4), 150 mM NaCl, 5 mM ethylenediaminetetra-acetic acid, 5% sodium dodecyl sulphate]. Nucleic acids were purified by four phenol chloroform (1:1) extractions, with elimination of double-stranded DNA by a final precipitation in 3M LiCl. Purity of rRNA samples was estimated from spectrophotometric absorbance ratios 260/280 = 2.00–2.15 and 230/280 ≤ 0.5. Integrity was verified by non-denaturing agarose gel electrophoresis. Sequence analysis was performed by comparison of the D2 variable domain of 26S rRNA, using the primer 5'GGTCCGTGTTTCAAGACGG(635).

For phylogenetic analysis, 21 sequenses of *Trichosporon* species published by Guého et al. (1992) were added (Fig. 2 and 5); seven of these are printed in Fig. 1 for comparison.

Sequence alignment and phylogenetic analysis

Analysis was undertaken using computer programs developed for PC-compatible computers by one of us (RC) and programs for molecular phylogeny

neighbor-joining (Saitou & Nei 1987), bootstrap, maximum likelihood (Felsenstein 1990) and PAUP (Swofford 1990).

For any phylogenetic analysis, species with large deletions, such as *Cryptococcus gastricus* and *Moniliella suaveolens*, were excluded. Furthermore it was sometimes unclear whether saturation of mutations was reached, i.e. if a difference observed between two sequences resulted from a single event or from several, superimposed mutations. In such cases several analyses were run, with and without these regions, and only those trees that showed consistent topology were retained.

Neighbor joining was used first, because it is extremely fast, even with large numbers of species. Each analysis was subsequently confirmed using maximum likelihood and maximum parsimony approaches. The latter was performed using the PAUP program with the 'branch and bound' option; it was followed by bootstrap analysis with a heuristic search and at least 100 replications. Some branches, identified in the most parsimonious tree but having low bootstrap percentages, have been omitted. Branches identified at a lower probability are not shown. Grouping of species was considered robust when identified by all three methods described above.

Secondary structure reconstruction

The secondary structure of the D2 domain around the deletion observed in the sequence of *C. gastricus* was obtained using the PC-FOLD program of Jaeger et al. (1990).

Results

A: Sequence diversity

The sequences of the variable domain D2 of LSU rRNA of 45 strains belonging to 43 species are shown in Fig. 1. Two hundred and ninety eight positions have been aligned, the first base read after the primer corresponding to position 626 (298 in Fig. 1) with reference to LSU rRNA primary structure of *Saccharomyces cerevisiae* (Georgiev et al. 1981).

The sequence of the type strain of *Cryptococcus neoformans (Filobasidiella neoformans)*, CBS 132 (serotype D) was used as model. Fig. 1 also includes a selection of sequences of *Trichosporon* species, representing different intra-generic branches published earlier by Guého et al. (1992). The sequences were arranged according to the order of the general phylogenetic analysis presented in Fig. 2.

Most sequences could be well aligned over the entire domain analyzed. The possibility of an alignment along the entire length of such a variable domain of the rRNA molecule suggests a recent separation among lineages of these organisms. Alignments of *Moniliella suaveolens* and *Trichosporonoides oedocephalis* were possible only in the 5' and 3' ends of the sequence (Fig. 1; only *M. suaveolens* shown). *Cryptococcus gastricus* was omitted from most phylogenetic analysis because this species had a large deletion of 33 nucleotides (Fig. 1).

The D2 variable domain allowed the separation of 3 out of 4 serotypes known in *C. neoformans*. The sequence closest to the model (var. *neoformans*, serotype D) was the one shared by serotypes B and C (var. *bacillispora*). The most distant sequence was that of *Moniliella suaveolens* with 148 substitutions out of 298 positions read, including a deletion of 44 nucleotides.

B: General phylogeny

The general tree obtained by neighbor-joining analysis is shown in Fig. 2. These results were verified using parsimony (with bootstrap) and maximum likelihood methods. Parsimony identified two trees, which differed from results of neighbor-joining only in small details. Bootstrap analysis confirmed the robustness of the same branches with percentages indicated in Fig. 2 above each solid branch. Maximum likelihood identified the same monophyletic groups, with internal branches statistically significant at $P < 0.01$, indicated in Fig. 2 by two asterisks below each branch. The major monophyletic groups (I, II and III in Fig. 2) are indicated by solid bars.

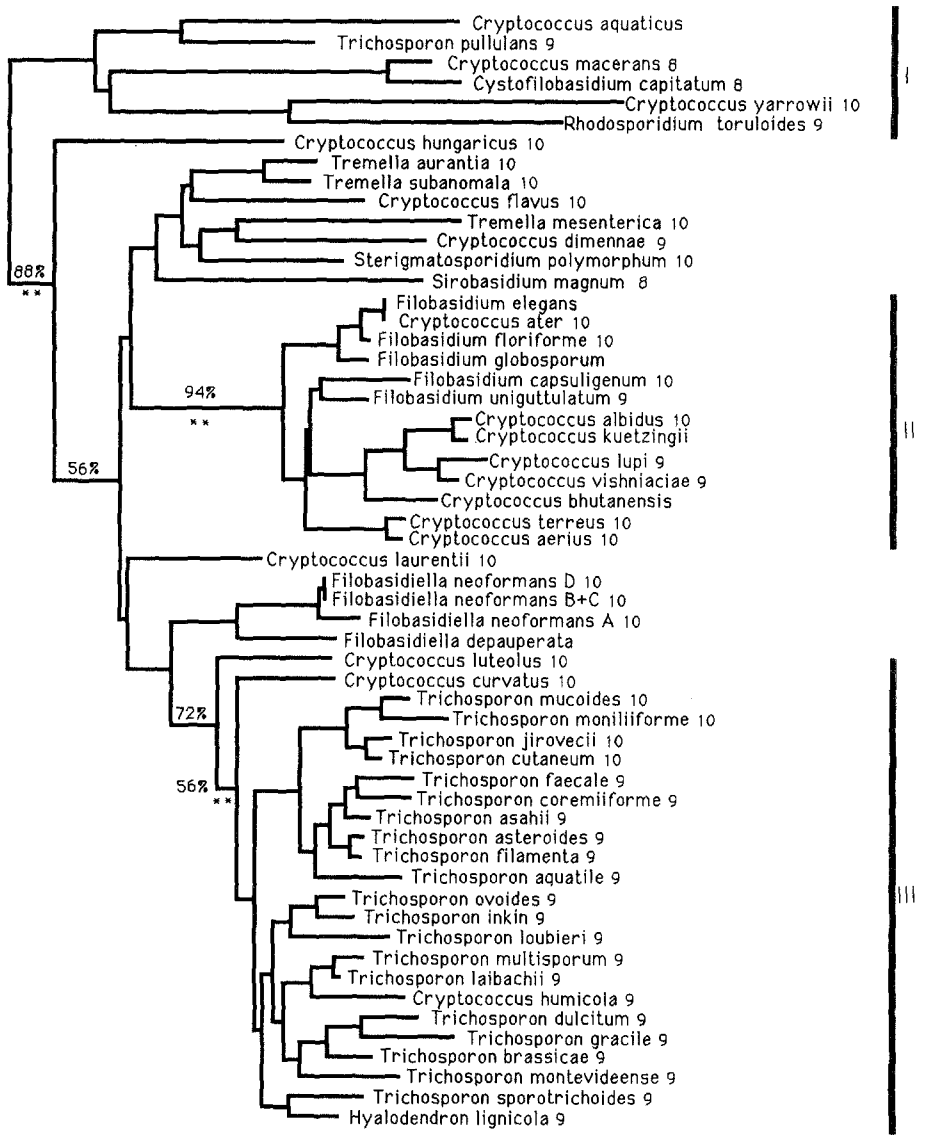

Fig. 2. General phylogeny of *Cryptococcus neoformans* and related species. Topology was obtained with the neighbor-joining method and confirmed by maximum parsimony (% of bootstrap replications indicated when compatible) and maximum likelihood (** indicate branches with $P < 0.01$). Vertical bars indicate groupings obtained. Group I contains outgroups species; groups II and III are robust monophyletic groups. Remaining species cannot be attributed to any robust taxon. Numbers indicate isoprenoid side chains of coenzyme Q systems found in the respective species.

Group I can be considered as comprising outgroup species, taking into account the large genetic distances separating each species from all the others. The group comprises three *Cryptococcus* species (*C. aquaticus*, *C. macerans* and *C. yarrowii*), as well as *Trichosporon pullulans*. Mutual distances within Group I being large, it is difficult to resolve precisely phylogenetic relationships within the group. The only robust relationship that can be deduced is between *C. macerans* and *Cystofilobasidium capitatum*.

Group II is a well individualized, robust monophyletic unit, including all *Filobasidium* species and a large number of *Cryptococcus* species. All *Trichosporon* species, with the exception of *T. pullulans* also cluster in a rather well defined unit (Group

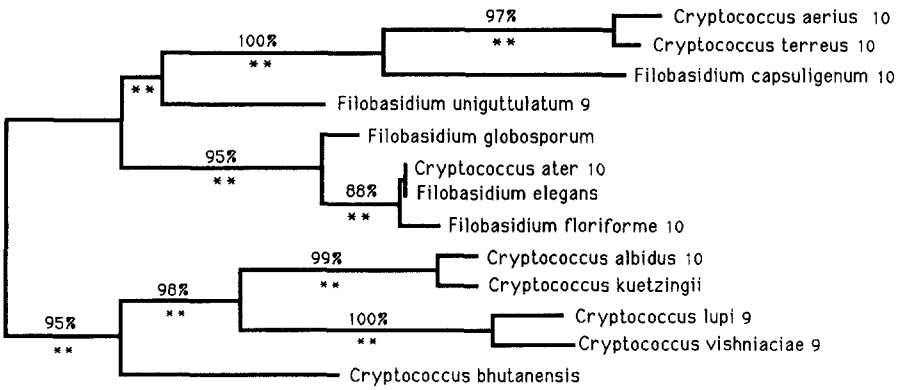

Fig. 3. Molecular phylogeny of *Filobasidium* and related species (Group II in Fig. 2). The tree is unrooted and constructed using methodologies applied in Fig. 2. Numbers indicate isoprenoid side chains of coenzyme Q systems found in the respective species.

III), which also includes some *Cryptococcus* species, in addition to *Hyalodendron lignicola*.

The phylogenetic relationships of the species not belonging to Groups I, II or III are unraveled with more difficulty, due to shortness of internodes. Only in a few cases it can be deduced with certainty that taxa are closely related species, e.g. *Tremella aurantia* and *T. subanomala* on the one hand and *Filobasidiella neoformans* (with its serotypes) and *F. depauperata* on the other.

The phylogenetic relationships within each group identified above were studied further by conducting more specific analyses that excluded distantly related taxa. This approach was necessary because of the presence of outgroups with positions that possibly mutated several times (mutation saturation), leading to distortions in tree topology (Smith et al. 1992).

C: Filobasidium and related taxa (Group II)

Bootstrap analysis of Group II (Fig. 3) revealed a number of significant pairings, viz. *Cryptococcus aerius* with *C. terreus*, *Filobasidium elegans* (including *C. ater* having the same sequence) with *F. floriforme* and this group with *F. globosporum*; *C. albidus* with *C. kuetzingii*, and *C. lupi* with *C. vishniaciae*. The association of the last four species in a monophyletic branch is supported.

When the sequence of *C. gastricus* was compared to those of *C. albidus* and *C. kuetzingii*, but excluding the portion containing a large deletion, the three species proved to be related, as already suspected from primary RNA structures (Fig. 1). Comparison of partial secondary structures of *C. albidus* and *C. gastricus* showed that the large deletion appearing in the latter species results from the disappearance of a small helix (33 nucleotides; A in Fig. 4). The remaining part of the sequence, including a second large helix (B in Fig. 4) and another deletion of 18 nucleotides (Fig. 1) is mostly similar in all three species concerned.

D: Trichosporon and related taxa (Group III)

A phylogenetic analysis of all species of Group III (Fig. 2) as well as of species not included in any of the numbered groups, is presented in Fig. 5. Several most parsimonious trees could be constructed, but the consensus tree was in good agreement with the topologies obtained by neighbor-joining and maximum likelihood. Several branches are supported by high percentages of bootstrap replications. *Trichosporon* was identified as a well-defined, robust taxon that also included *Hyalodendron lignicola*, *Cryptococcus curvatus* and *C. humicola*, but not *C. luteolus*. Within this group, topologies were less consistent, because mutual similarities were too high. With this more limited set of species, *C. luteolus* was found well apart from *Trichosporon*.

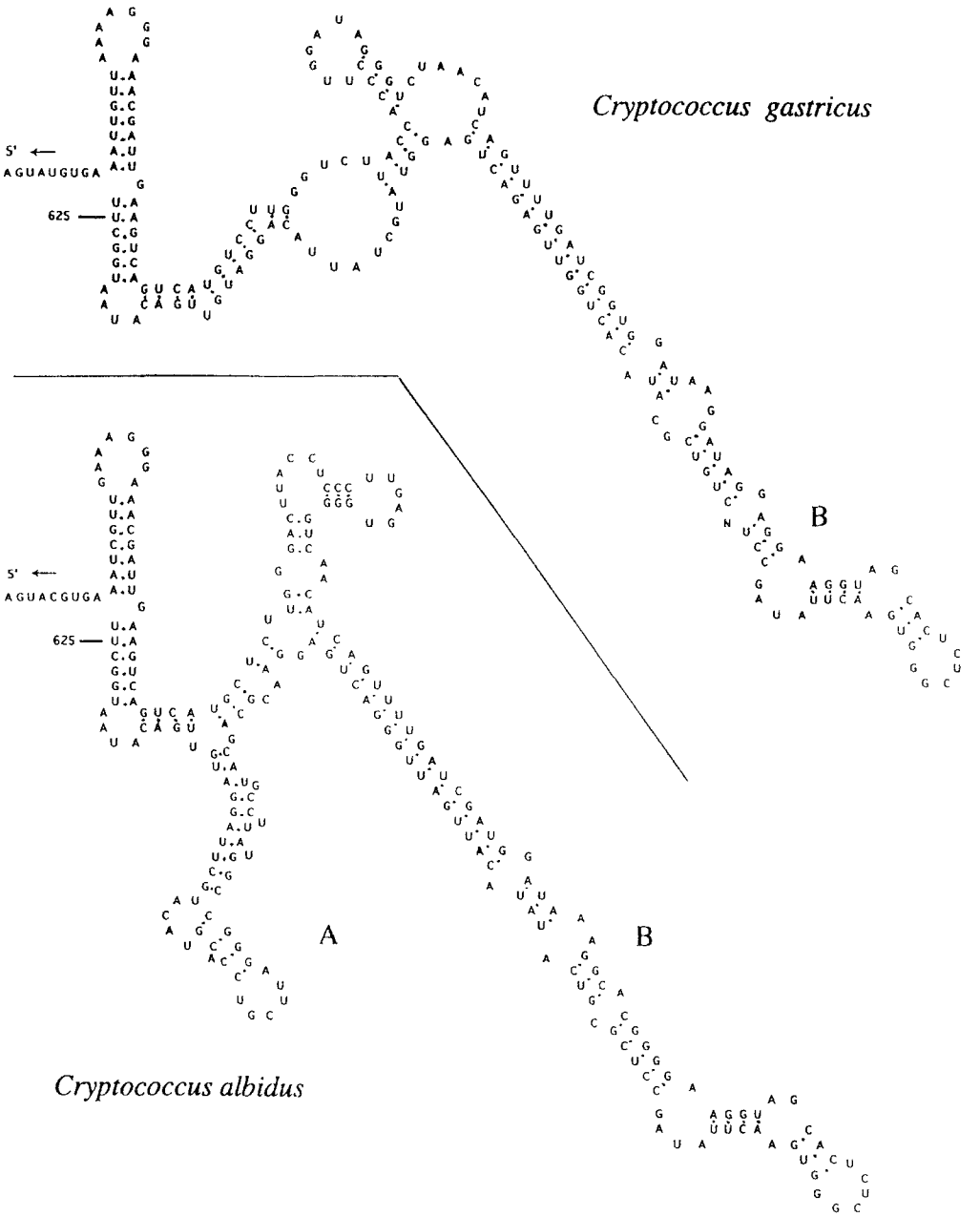

Fig. 4. Secondary structures of LSU rRNA D2 variable domain of *Cryptococcus gastricus* and *C. albidus*. A indicates a small helix which is missing in *C. gastricus*. Helix B shows strong similarity in both species.

E: Ambiguously positioned species

A precise determination of phylogenetic relations of the remaining species (Fig. 2) was difficult with all three approaches. This problem may be due to the presence of long peripheral branches with short internodes (too few species analyzed or analyzed domain too variable). A parsimony analysis using the most conserved parts of the domain led to 24 most parsimonious trees, with few branches identified in consensus. Exclusion of the distantly related *Trichosporon* species from the analysis (Fig. 6)

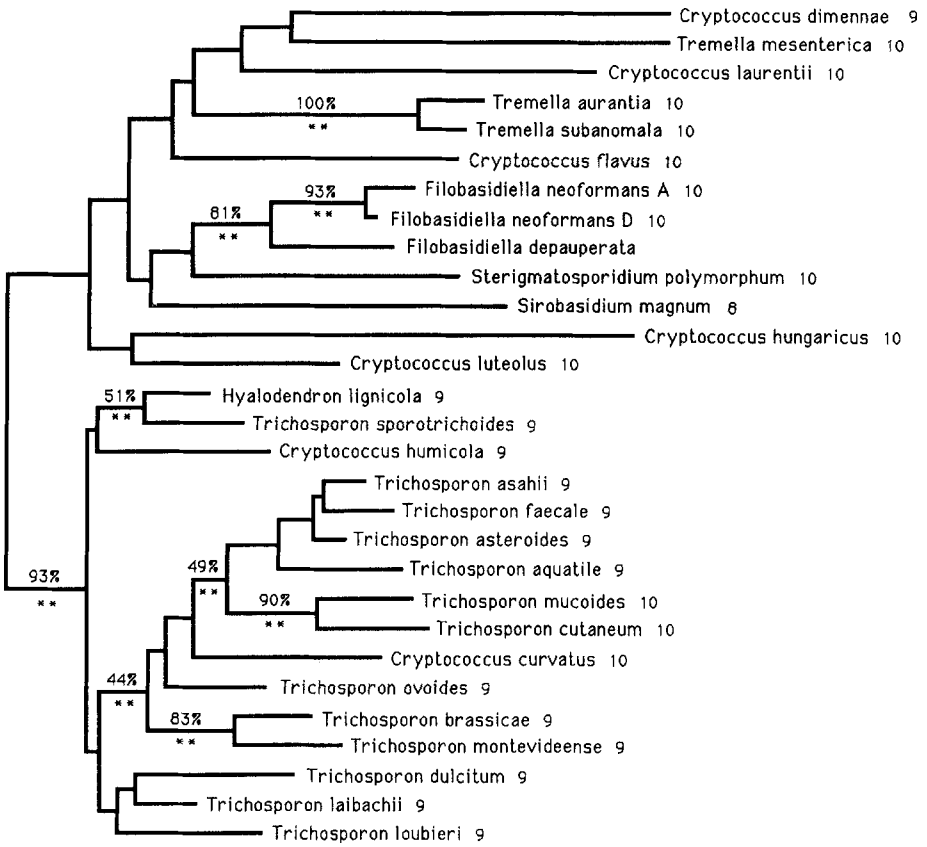

Fig. 5. Molecular phylogeny of *Trichosporon* (group III) and species resolved with difficulty in Fig. 2. The tree is unrooted and constructed using methodologies applied in Fig. 2. Numbers indicate isoprenoid side chains of coenzyme Q systems found in the respective species.

failed to improve the resolution of relationships. However, two robust groups were obtained, namely in *Filobasidiella* and the pair *T. aurantia / T. subanomala* which was found in all analyses and in 100% of the bootstrap replicates. In all analyses, the *Filobasidiella* species consistently clustered together. The *F. neoformans* serotypes were found to be closely related (Fig. 7).

Discussion

Taxa found to be closely related on the basis of rRNA sequences generally proved to have identical Co-Q systems. However, within robust groups such as *Trichosporon* or *Filobasidium*, two Co-Q systems may occur. This further underlines that this character cannot be used as an exclusive generic character (Guého et al. 1992)

Group III comprises all *Trichosporon* species, with the exception of *T. pullulans* which is identified as completely unrelated. Fell et al. (1992), using the same variable region of the LSU rRNA for a phylogenetic comparison of marine yeasts, found *T. pullulans* to be related to *Mrakia*, not too far from the genus *Cystofilobasidium*, as shown previously (Guého et al. 1989). The yeasts belonging to the genus *Mrakia* (probably a single taxon; Fell et al. 1992) are psychrophilic and are able to filament, as *T. pullulans*. However, they possess a Q8 ubiquinone system, as does *Cystofilobasidium*, but unlike *T. pullulans*, which has Q9. The homogeneity of the core of the genus is in agreement with earlier data presented by Guého et al. (1992) and is confirmed by the present comparison with more divergent basidiomycetous yeasts.

In contrast to *Trichosporon*, species hitherto classified in *Cryptococcus* are distributed all over the

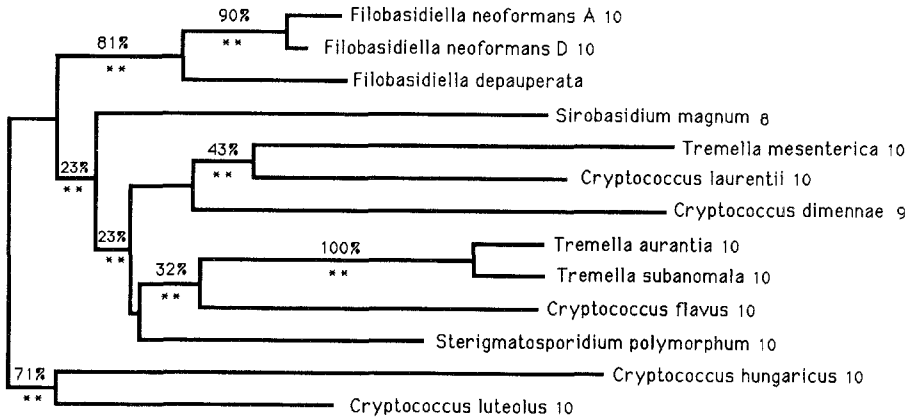

Fig. 6. Molecular phylogeny of species not assigned to any robust group in Fig. 2. The tree is unrooted and constructed using methodologies applied in Fig. 2. The association between *C. luteolus* and *C. hungaricus* is probably artefactual, because these species cluster close to a possible rooting. Numbers indicating isoprenoid side chains of coenzyme Q systems found in the respective species.

phylogenetic tree (Fig. 2). This demonstrates that this anamorph-genus is remarkably heterogeneous, as supposed earlier by Kocková-Kratochvílová et al. (1976) and Weijman et al. (1988). *Cryptococcus curvatus* and *C. humicola* are placed close to or amidst *Trichosporon* species, although they lack arthroconidia. All analyzed strains of *C. curvatus* came from humans, which is in accordance with the majority of *Trichosporon* species being human-pathogenic (Guého et al. 1992). The geophilic species *C. humicola* clusters with the few *Trichosporon* species which are mainly found in soil. With a different set of heterobasiomycetous yeasts, Fell et al. (1992) also found the two species to cluster with *Trichosporon*.

Several *Cryptococcus* species, such as *C. aquaticus* and *C. yarrowii*, are grouped in a heterogeneous assemblage of unrelated taxa interconnected by long branches. The heterogeneity of this group is underlined by the presence of three different coenzyme Q systems. *Cryptococcus macerans* clusters with *Cystofilobasidium capitatum*. The two species have pink colonies, are physiologically similar, particularly in the ability to assimilate inositol, have a Q8 ubiquinone and are unable to grow at temperatures above 20 °C. The teleomorph of *C. macerans* is as yet unknown, but will probably turn out to be a *Cystofilobasidium* species. *Leucosporidium larimarini* also belongs to this genus, as demonstrated by Fell et al. (1992) on the basis of LSU rRNA sequences.

The genus *Filobasidium* constitutes a robust, monophyletic group of species, which also includes *C. albidus*, *C. kuetzingii*, *C. gastricus*, *C. lupi*, *C. vishniaciae*, *C. bhutanensis*, *C. terreus*, *C. aerius* and *C. ater*. *Filobasidium* is characterized by holobasidia-producing groups of sessile, non-catenate basidiospores. The two recently described species, *F.*

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                    91
F.neoformans-D      CAGCCAGUUC  UGCUGGUGUA  UUCCCUUUAG  A*CGGGUCAA  CAUCAGUUCU
F.neoformans-BC     .......C..  ..........  ..........  ..........  ..........
F.neoformans-A      ..........  ..........  ..........  ..........  ..........
F.depauperata       ..........  ..........  ..........  ..........  ..........

                    141
F.neoformans-D      GAU*CGGUGG  AUAAGGGCUG  GAGGAAUGUG  GCACU****C  UUCGGGGUGU
F.neoformans-BC     ..........  ..........  ..........  ..........  ..........
F.neoformans-A      ..........  ..........  .G.......A  ..........  .....A....
F.depauperata       ..C.......  .A..A...A.  ......A...  A...C....U  C.........
```

Fig. 7. Alignment of LSU rRNA partial sequences of the D2 variable domain (5' to 3' direction) of serotypes of *Filobasidiella neoformans* and *F. depauperata*, with *F. neoformans* serotype D as model. Dashes are used when a particular position is identical to the nucleotide of *F. neoformans-D*; * represent deletions necessary for alignment. For a given serotype, all strains analyzed had identical sequences.

elegans and *F. globosporum* (Bandoni et al. 1991), show the same morphological characters and indeed cluster with the other *Filobasidium* species. *C. ater* has been found to be identical to *F. elegans* and thus might represent its anamorph. However, it produces dark pigments which are absent from *F. elegans*; a definitive conclusion on its relationship is as yet impossible.

The group *Cryptococcus terreus* / *C. aerius* clusters closely with *Filobasidium* species. *C. aerius* has been treated as a variety of *C. albidus* (Phaff & Fell 1970), but that species has a quite different partial rRNA sequence. The large genetic distance observed between the two taxa is in agreement with the data given by Yamazaki & Komagata (1982), who found markedly dissimilar electrophoretic protein patterns in the two species. Vaughan Martini (1991) and Sugita et al. (1992) found the species to differ on the basis of nDNA/DNA reassociations.

The supposition that *C. albidus* var. *albidus* might be the anamorph of *Filobasidium floriforme* (Moore & Kreger-van Rij 1972) is refuted by large differences in partial 26S rRNA sequences. *Cryptococcus kuetzingii* and *C. albidus* have been regarded as closely related and have been synonymized on the basis of enzyme profiles (Yamazaki & Komagata 1982). Partial RNA sequences of *C. kuetzingii*, CBS 6086 appeared identical to *C. albidus*, but the type strain, CBS 1926, is slightly different.

Cryptococcus gastricus is clearly distinct from *C. albidus* and similar species, but this is mainly due to a large deletion of 33 nucleotides (Fig. 1). The secondary structure of the adjacent parts of the rRNA molecule shows that a loss of a helix is concerned (A in Fig. 4), which is probably a unique event. A phylogenetic analysis excluding the domain of the deletion shows that the species indeed cluster together, not too far from *C. lupi* (Fig. 3). This is confirmed by the observation that *C. gastricus* shares another deletion of 15 nucleotides with *C. albidus* and *C. kuetzingii*, as found earlier by Fell et al. (1992).

The psychrophilic soil species *C. vishniaciae* and *C. lupi* should be regarded as close relatives, as supposed by Baharaeen & Vishniac (1982). Fell et al. (1992) found *C. lupi* to be identical to a number of varieties of *C. vishniaciae*, but did not include the typical variety.

Cryptococcus laurentii is known as a heterogeneous amalgamate. Mol% G+C of DNA varies from 51 to 59% (Nakase & Komagata 1971). The two mating types (NRRL Y-7313 and Y-7139; Kurtzman 1973) have the same partial rRNA sequences but differ in 10 bases from the type, CBS 139. Presence of tremelloid dolipores has been reported in *C. laurentii* by Rhodes et al. (1981). Slodki et al. (1966) regarded the species as being of heterobasidiomycetous relationship on the basis of similarities between extracellular polysaccharides of certain *Tremella* species and *C. laurentii*. CBS 139 clustered relatively close to *Tremella aurantia* and *T. subanomala*, confirming isoenzyme similarities found by Yamazaki & Komagata (1982). Kurtzman (1973) noted a similarity between the yeast forms of *T. aurantia* and *C. laurentii*, but was unable to cross strains of *T. aurantia* with any of the mating types Y-7313 and Y-7139.

Cryptococcus flavus was previously classified in *Rhodotorula* because of its yellowish colony colour (Lodder 1934). In most analyses it clustered together with *C. dimennae*, among members of the Tremellales (Figs 2, 3 and 4). However, very few tremellaceous fungi have as yet been sequenced, and this renders conclusions on relationship somewhat premature. *Tremella aurantia* and *T. subanomala* are able to assimilate inositol, whereas *T. mesenterica* is not (Rodrigues de Miranda & Bandoni 1984). As *Tremella* species are primarily distinguishable on the basis of this physiological difference, it would be interesting to assess the importance of this character from a phylogenetic point of view.

The teleomorph-genus *Filobasidiella* seems to form a rather well-delimited group. *F. depauperata*, which lacks a yeast-like anamorph and clamp connections (Samson et al. 1983), clusters together with *F. neoformans*. Nevertheless, the two species are connected by long branches. *Filobasidiella* is found fairly close to *Trichosporon*.

Filobasidiella has non-septate basidia producing chains of basidiospores. No *Cryptococcus* species other than *C. neoformans*, the anamorph of *Filobasidiella neoformans*, is found in this group. Fell et al. (1992) found *Tsuchiyaea wingfieldii* and *Cryptococcus amylolentus* to be relatively closely related. *C. neoformans* exhibits a neurotropic pathology in

warm-blooded animals. In humans its behaviour is remarkable by showing an association with impaired T-cell immunity. The large phylogenetic distance from other *Cryptococcus* species might explain why these are rarely found as medically significant agents. When found, they probably take advantage of impaired innate rather than acquired immunity, similar to the great majority of opportunistic fungi. *Cryptococcus luteolus* was found by Yamazaki & Komagata (1982) to be similar to *C. neoformans* in nutritional physiology and isoenzyme patterns. It is medically insignificant because of its incapacity to grow at 37 °C.

A rather large series of the four serotypes of *Filobasidiella neoformans* were analyzed (4 strains of serotypes A and C and 5 of B and D, respectively). All four were found to be identical when parts of 18S and 26S were sequenced with primers 5'-ACGGGCGGTGTGTAC and 5'-TTGGAGACCTGCTGCGG, respectively (Guého et al. 1989). Differences were found, however, when the variable portion of the 26S large subunit of rRNA was used starting from position 626. With this partial sequence of 298 nucleotides, three differences were found between serotypes A and D, four between A and B and one between B and D; serotype C was identical to B (Fig. 7). From these data serotype D appears phylogenetically closer to B and C than to A. Mating partners belonging to serotypes B or C give rise to a teleomorph belonging to var. *bacillispora*. The phylogenetic distance between serotypes A and D, however, probably excludes the possibility of sexual compatibility. Mating between partners of serotype D alone have resulted in a teleomorph of var. *neoformans*. The progeny is supposed to be viable. The original description of *Filobasidiella neoformans* var. *neoformans* was based on a pairing between two strains of serotype D (Kwon-Chung 1975). Progeny of partners of A and D, however, is not viable (Kwon-Chung, pers. comm.). Combinations of suitable partners belonging to other serotypes at the most resulted in teleomorphs with aberrant morphology, as may be judged from photographs published by Schmeding et al. (1981). Our own observation of mating between A and B serotypes confirmed this irregular morphology. However, DNA comparison between strains of serotypes D and A results in very high percentages of relatedness (88–94%), while DNA homology is intermediate (55–63%) when crossing D or A with B or C (Aulakh et al. 1981). More genetic studies should be done to understand clearly the relationships between serotypes and mating types.

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