

Molecular Phylogeny of the Genus *Cryptococcus* and Related Species Based on the Sequences of 18S rDNA and Internal Transcribed Spacer Regions

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The 18S rDNA sequences of type strains of 33 species and several reference strains of the genus *Cryptococcus* were determined and the phylogenetic relationships among them and related basidiomycetes including yeasts were analyzed by the neighbor-joining method and the maximum parsimony method. For the phylogenetically closely related species revealed from 18S rDNA sequence analyses, the sequences of the internal transcribed spacer (ITS) regions were also determined and compared pairwisely. *Cryptococcus* species, except for *C. yarrowii*, were divided into two main groups. Five species, *C. aquaticus*, *C. curiosus* (synonym of *Mrakia frigida*), *C. huempii*, *C. feraegula* and *C. macerans*, showed a close relationship with Cystofilobasidiales. Twenty-three species were included in five respective lineages (*Bullelomyces* lineage, *Filobasidiella* lineage, *Filobasidium* lineage, *C. humicolus-Trichosporon* lineage and *C. luteolus* lineage) in Filobasidiales and Tremellales with species of other genera. The remaining five species showed affinity to the group of Filobasidiales and Tremellales, but were believed not to belong to these five lineages based on the topologies of phylogenetic trees and the results of bootstrap analyses. The sequence comparison of ITS regions indicated the following extremely closely related species : *C. albidus* JCM 2334 (type strain of *C. albidus*) and *C. kuetzingii*; *C. albidus* JCM 3688 (formerly type strain of *C. albidus* var. *diffluence*) and *C. albidosimilis*; *C. antarcticus* and *C. bhutanensis*; *C. ater* and *C. magnus*; *C. consortions* and *C. vishniacii*; *C. gastricus* and *C. gilvescens*; and *C. fuscensens* and *C. terreus*. *C. yarrowii* showed a urediniomycetous affinity.

Key words : *Cryptococcus*, phylogeny, 18S rDNA, internal transcribed spacer (ITS)

INTRODUCTION

The genus *Cryptococcus* is a basidiomycetous anamorphic yeast. No living culture of the type species of this genus, *C. mollis*, was preserved, however, Rodrigues de Miranda and Batenburg-van der Verte (17) observed the existence of basidiomycetous yeasts in the type specimen using an electron microscope. Based on these facts, Fell et

al.(2) proposed to conserve *Cryptococcus* with *C. neoformans* (Sanfelice) Vuillemin as the neotype species. Many authors studied the taxonomy of this genus, and the boundaries between *Cryptococcus* and others were changed several times (4). Weijman et al.(29) emended the genera *Candida*, *Cryptococcus* and *Rhodotorula* based on the affinitive characters for Ascomycetes or Basidiomycetes, and distinguished the latter two genera by the presence or absence of xylose in the cells. The presence of dolipores in the hyphal septa of *Filobasidiella neoformans*, teleomorph of *C. neoformans*, and *C. laurentii* indicated the close relationship with Hymenomycetes (11, 15). In "The Yeasts, A Taxonomic Study", 4th ed., 34 species are recognized in

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the genus (4). The heterogeneity of this genus has been reported based on whole cell protein patterns, DNA base compositions, major ubiquinones and sequence analyses of 5.8S rDNA, the part of 18S rRNA and/or 26S rRNA, and D1/D2 region of 26S rDNA (1, 7, 12, 13, 28, 31, 32). A phylogenetic approach also showed that ballistoconidia-forming yeasts and stalked conidia-forming yeasts were closely related to the genus *Cryptococcus* (23~25). Recently, Fell et al. (3) proposed a new order, Cystofilobasidiales, in the class Hymenomycetes and reported that *C. aquaticus*, *C. feraegula* and *C. macerans* showed a very close relationship with Cystofilobasidiales based on partial sequence of 26S rDNA. In this study, we determined the sequences of 18S rDNA of type strains of 33 species and some reference strains of the genus *Cryptococcus* and analyzed the phylogenetic relationship among them and related basidiomycetes including yeasts. For the phylogenetically closely related species based on 18S rDNA sequences, the sequences of internal transcribed spacer (ITS) regions were also determined and pairwisely compared.

MATERIALS AND METHODS

Strains used. The strains used for sequencing are shown in Table 1 with DDBJ accession numbers of the sequence data of 18S rDNA and ITS regions.

Sequencing and phylogenetic analysis. The nucleotide sequences of 18S rDNA and ITS regions (ITS1 and 2) including the 5.8S rDNA were directly determined using PCR products according to the method of Sugita and Nakase (19). The amplified PCR products were purified using E. Z. N. A. Cycle-Pure Kit (Omega Biotek, USA) according to the manufacturer's instruction. Direct sequencing of the PCR products was performed with an ABI PRISM Cycle sequencing kit and a 5X Sequence Buffer (Perkin-Elmer Applied Biosystems, USA). The completed sequencing reactions were electrophoresed on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems). Generated 18S rDNA sequences were aligned with related species using the CLUSTAL W ver. 1.74 computer program (26). Reference sequences used for the phylogenetic study were obtained from the database (Table 2). Two kinds of methods in the PHYLIP Version 3.57c computer program (6), the neighbor-joining method with evolutionary distance and the maximum parsi-

mony method, were employed to construct phylogenetic trees. The evolutionary distance was calculated according to Kimura (9). Sites where gaps existed in any sequences were excluded. Bootstrap analyses (5) were performed from 100 random resamplings for both methods. For the comparison of ITS regions among closely related species, sequences were aligned by eye, and the sequence similarity including gaps was pairwisely calculated.

RESULTS AND DISCUSSION

18S rDNA sequences determined in this study were aligned well with other related basidiomycetes including yeasts. Phylogenetic trees constructed by the neighbor-joining method (Fig. 1) and the maximum parsimony method (Fig. 2) clearly showed the heterogeneity of the genus *Cryptococcus*. They occurred at every cluster of the trees and constituted branches with other genera. *C. yarrowii* showed a urediniomycetous affinity. These topologies are the same as the reported trees (1, 3, 7). *Cryptococcus* species, except for *C. yarrowii*, were divided into two main groups. Five species, *C. aquaticus*, *C. curiosus*, *C. huempii*, *C. feraegula* and *C. macerans*, showed close relationship with Cystofilobasidiales. *C. curiosus* was treated as a synonym of *M. frigida* in "The Yeasts, A Taxonomic Study", 4th ed.(4). *C. aquaticus* was clustered with *Mrakia frigida*, and *C. feraegula* and *C. macerans* were closely related to *Cystofilobasidium capitatum*. *C. huempii* constituted a cluster with *C. aquaticus* and *Mrakia frigida* in the maximum parsimony tree (Fig. 2), and was located at the contiguous position of the species of Cystofilobasidiales including *Xanthophyllomyces dendrophous* and *Trichosporon pullulans* in the neighbor-joining tree (Fig. 1). *C. huempii* was not examined in the tree of Fell et al.(3), however, its phylogenetic position and possession of Q-8 as a major ubiquinone (CBS database) of this species would be proof of a close relationship to Cystofilobasidiales. Our results also indicated that *X. dendrophous* should be transferred from Filobasidiales to Cystofilobasidiales, although this species was not included in Cystofilobasidiales in the paper by Fell et al.(3).

The remaining species were positioned in the group of Filobasidiales and Tremellales and mainly included in the five lineages : *Bullelomyces* lineage, *Filobasidiella* lineage, *Filobasidium* lineage, *C.*

Table 1. Strains used in this study

Scientific name	JCM no.	Other designations	DDBJ accession numbers	
			18 S rDNA	ITS & 5.8 S
Genus <i>Cryptococcus</i>				
<i>C. aerius</i>	10481 T	=ATCC 10665=CBS 155=NRRL Y-1399	AB032614	AB032666
<i>C. albidosimilis</i>	8843 T	=ATCC 76863=NRRL Y-17463	AB032615	AB032667
<i>C. albidus</i>	2334 T	=IFO 0610	AB032616	AB032668
<i>C. albidus</i> (= <i>C. albidus</i> var. <i>diffluens</i>)	3688 T of <i>C. albidus</i> var. <i>diffluens</i>	=CBS 160	AB032617	AB032669
<i>C. amylorentus</i>	1690 T	=CBS 6039	AB032619	
<i>C. antarcticus</i>	8844 T	=ATCC 76663=NRRL Y-17461	AB032620	AB032670
<i>C. aquaticus</i>	1775 T	=CBS 5443	AB032621	
<i>C. ater</i>	10451 T	=ATCC 14247=CBS 4685=IFO 1862	AB032622	AB032671
<i>C. bhutanensis</i>	9424 T	=CBS 6294	AB032623	AB032672
<i>C. cellulolyticus</i>	9707 T	=CBS 8294	AB032624	
<i>C. consortium</i>	9425 T	=ATCC 56686=CBS 7159	AB032625	AB032673
<i>C. curvatus</i>	1532 T	=ATCC 10567=CBS 570=NRRL Y-1511	AB032626	
<i>C. dimennae</i>	8974 T	=CBS 5770=MUCL 30443	AB032627	
<i>C. feraeugula</i>	10475 T	=CBS 7202=VKM Y-2856	AB032628	
<i>C. flavus</i>	8332 T	=IFO 0407	AB032629	
<i>C. friedmannii</i>	9426 T	=ATCC 56687=CBS 7160	AB032630	AB032674
<i>C. fuscescens</i>	9427 T	=CBS 7189	AB032631	AB032675
<i>C. gastricus</i>	3691 T	=CBS 1927=UCD 68-185	AB032632	AB032676
<i>C. gastricus</i>	3692		AB032633	AB032677
<i>C. gilvescens</i>	9884 T	=CBS 7525=VKM Y-2748	AB032634	AB032678
<i>C. heveanensis</i>	3693 T	=CBS 569=UCD 68-215	AB032635	
<i>C. huempfii</i>	9429 T	=CBS 8186	AB032636	
<i>C. humiculus</i>	1457 T	=CBS 571	AB032637	
<i>C. hungaricus</i>	9046 T	=IFO 1052	AB032638	
<i>C. kuetzingii</i>	9037 T	=CBS 1926=IFO 1866	AB032639	AB032679
<i>C. laurentii</i>	9066 T	=CBS 139=IFO 0609	AB032640	
<i>C. luteolus</i>	3689 T	=CBS 943	AB032641	
<i>C. macerans</i>	9048 T	=CBS 2206=IFO 1870	AB032642	
<i>C. magnus</i>	9038 T	=CBS 140=IFO 0698	AB032643	AB032680
<i>C. marinus</i>	9885 T	=CBS 5235	AB032644	
<i>C. podzolicus</i>	10452 T	=ATCC 34208=CBS 6819=MUCL 29872	AB032645	
<i>C. skinneri</i>	9039 T	=CBS 5029=IFO 1872	AB032646	
<i>C. terreus</i>	10453 T	=ATCC 11799=CBS 1895=MUCL 30418	AB032647	AB032681
<i>C. terreus</i> (= <i>C. elinovii</i>)	8975 T of <i>C. elinovii</i>	=CBS 7051=MUCL 30457	AB032648	AB032682
<i>C. terreus</i> (= <i>C. himalayaensis</i>)	9493 T of <i>C. himalayaensis</i>	=IAM 4963	AB032649	AB032683
<i>C. vishniacii</i>	10482 T	=ATCC 36649=CBS 7110=NRRL Y-17208	AB032650	AB032684
<i>C. vishniacii</i> (= <i>C. asgardensis</i>)	9422 T of <i>C. asgardensis</i>	=ATCC 46399=CBS 8141	AB032651	AB032685
<i>C. vishniacii</i> (= <i>C. baldrensis</i>)	9423 T of <i>C. baldrensis</i>	=ATCC 46400=CBS 8142	AB032652	AB032686
<i>C. vishniacii</i> (= <i>C. hempflingii</i>)	9428 T of <i>C. hempflingii</i>	=ATCC 46401=CBS 8143	AB032653	AB032687
<i>C. vishniacii</i> (= <i>C. lupi</i>)	9047 T of <i>C. lupi</i>	=ATCC 44529=IFO 10127	AB032654	AB032688
<i>C. vishniacii</i> (= <i>C. socialis</i>)	9430 T of <i>C. socialis</i>	=ATCC 56685=CBS 7158	AB032655	AB032689
<i>C. vishniacii</i> (= <i>C. tyrolensis</i>)	9431 T of <i>C. tyrolensis</i>	=ATCC 46405=CBS 8144	AB032656	AB032690
<i>C. vishniacii</i> (= <i>C. vishniacii</i> var. <i>asocialis</i>)	9049 T of <i>C. vishniacii</i> var. <i>asocialis</i>	=ATCC 46402=IFO 10128	AB032657	AB032691
<i>C. yarrowii</i>	8232 T	=IGC 4525	AB032658	
Genus <i>Fellomyces</i>				
<i>F. borneensis</i>	10163 T	=CBS 8282	AB032659	
<i>F. chinensis</i>	10164 T	=CBS 8278	AB032660	
<i>F. lichenicola</i>	10165 T	=CBS 8315	AB032661	
<i>F. sichuanensis</i>	10166 T	=CBS 8318	AB032662	
<i>F. thailandicus</i>	10167 T	=CBS 8308	AB032663	
Genus <i>Filibasidium</i>				
<i>F. uniguttulatum</i>	3685 T	=CBS 1730=IFO 0699	AB032664	AB032692
(Anamorph : <i>C. uniguttulatus</i>)				
Genus <i>Mrakia</i>				
<i>M. frigida</i> (= <i>C. curiosus</i>)	1596 T of <i>C. curiosus</i>	=ATCC 18816=CBS 5688=IFO 1336	AB032665	

T, Type strain

Table 2. 18S rDNA sequence data obtained from the databank for constructing the phylogenetic tree

Scientific name	DDBJ/EMBL/Gen Bank No.	Scientific name	DDBJ/EMBL/Gen Bank No.
<i>Atheria bombacina</i>	M55638	<i>Kockovaella schimae</i>	AB005482
<i>Auricularia auricula-judae</i>	L22254	<i>Kockovaella thailandica</i>	D64133
<i>Bullera armeniaca</i>	D78323	<i>Mrakia frigida</i>	D12802
<i>Bullera coprosmaensis</i>	D78326	<i>Sirobasidium magnum</i>	AF053717
<i>Bullera crosea</i>	D31648	<i>Spongipellis unicolor</i>	M59760
<i>Bullera dendrophila</i>	D31649	<i>Sporidiobolus johnsonii</i>	L22261
<i>Bullera globispora</i>	D31650	<i>Sterigmatosporidium polymorphum</i>	D64120
<i>Bullera hannaë</i>	D78327	<i>Tremella foliacea</i>	L22262
<i>Bullera huiaensis</i>	D78331	<i>Tremella globospora</i>	U00976
<i>Bullera miyagiana</i>	D31651	<i>Tremella moriformis</i>	U00977
<i>Bullera mirakii</i>	D78325	<i>Trichosporon aquatile</i>	AB001730
<i>Bullera oryzae</i>	D31652	<i>Trichosporon asahii</i>	AB001726
<i>Bullera pennisetica</i>	AB005452	<i>Trichosporon asteroides</i>	AB001729
<i>Bullera pseudoalba</i>	D31660	<i>Trichosporon brassicae</i>	AB001731
<i>Bullera sinensis</i>	D78328	<i>Trichosporon coremiiforme</i>	AB001727
<i>Bullera unica</i>	D78330	<i>Trichosporon cutaneum</i>	AB001753
<i>Bullera variabilis</i>	D31654	<i>Trichosporon domesticum</i>	AB001754
<i>Bulleromyces albus</i>	X60179	<i>Trichosporon dulcitum</i>	AB001755
<i>Calocea cornea</i>	L22256	<i>Trichosporon faecale</i>	AB001728
<i>Cryptococcus neoformans</i> serotype B	L05427	<i>Trichosporon gracile</i>	AB001756
<i>Cryptococcus neoformans</i> serotype D	L05428	<i>Trichosporon inkin</i>	AB001757
<i>Cystofilobasidium capitatum</i>	D12801	<i>Trichosporon japonicum</i>	AB001749
<i>Dacrymyces chrysospermus</i>	L22257	<i>Trichosporon jirovecii</i>	AB001758
<i>Fellomyces distylii</i>	AB001036	<i>Trichosporon laibachii</i>	AB001760
<i>Fellomyces fuzhouensis</i>	AB001032	<i>Trichosporon loubieri</i>	AB001759
<i>Fellomyces horovitziae</i>	AB001033	<i>Trichosporon moniliiforme</i>	AB001761
<i>Fellomyces ogasawarensis</i>	AB001035	<i>Trichosporon montevideense</i>	AB001762
<i>Fellomyces penicillatus</i>	AB001034	<i>Trichosporon mucoides</i>	AB001763
<i>Fellomyces polyborus</i>	D64117	<i>Trichosporon ovoides</i>	AB001765
<i>Fibulobasidium inconspicuum</i>	D64123	<i>Trichosporon pullulans</i>	AB001766
<i>Filobasidiella neoformans</i>	D12804	<i>Trichosporon sporotrichoides</i>	AB001767
<i>Filobasidium floriforme</i>	D13460	<i>Tsuchiyaea wingfieldii</i>	D64121
<i>Holtermannia corniformis</i>	AF053718	<i>Udeniomyces megalosporus</i>	D31657
<i>Kockovaella imperatae</i>	AB005561	<i>Udeniomyces pyricola</i>	D31659
<i>Kockovaella machilophila</i>	AB005479	<i>Udeniomyces puniceus</i>	D31658
<i>Kockovaella phaffii</i>	AB005480	<i>Xanthophyllomyces dendrorhous</i>	D31656
<i>Kockovaella sacchari</i>	AB005453		

humicolus-*Trichosporon* lineage and *C. luteolus* lineage (Figs. 1 and 2, and Table 3). Five species, *C. dimennae*, *C. flavus*, *C. marinus*, *C. podzolicus* and *C. skinneri*, were considered not to belong to these lineages based on the topologies of phylogenetic trees and bootstrap values. *C. dimennae* and *C. skinneri* clustered with *Bullera globispora* and

Tremella foliacea, respectively. However, the phylogenetic positions of these five *Cryptococcus* species were changeable depending on their alignment and/or the methods of phylogenetic analyses as shown in Figs. 1 and 2. Further sequencing study including *Tremella* species will be necessary to determine the phylogeny of these five species.

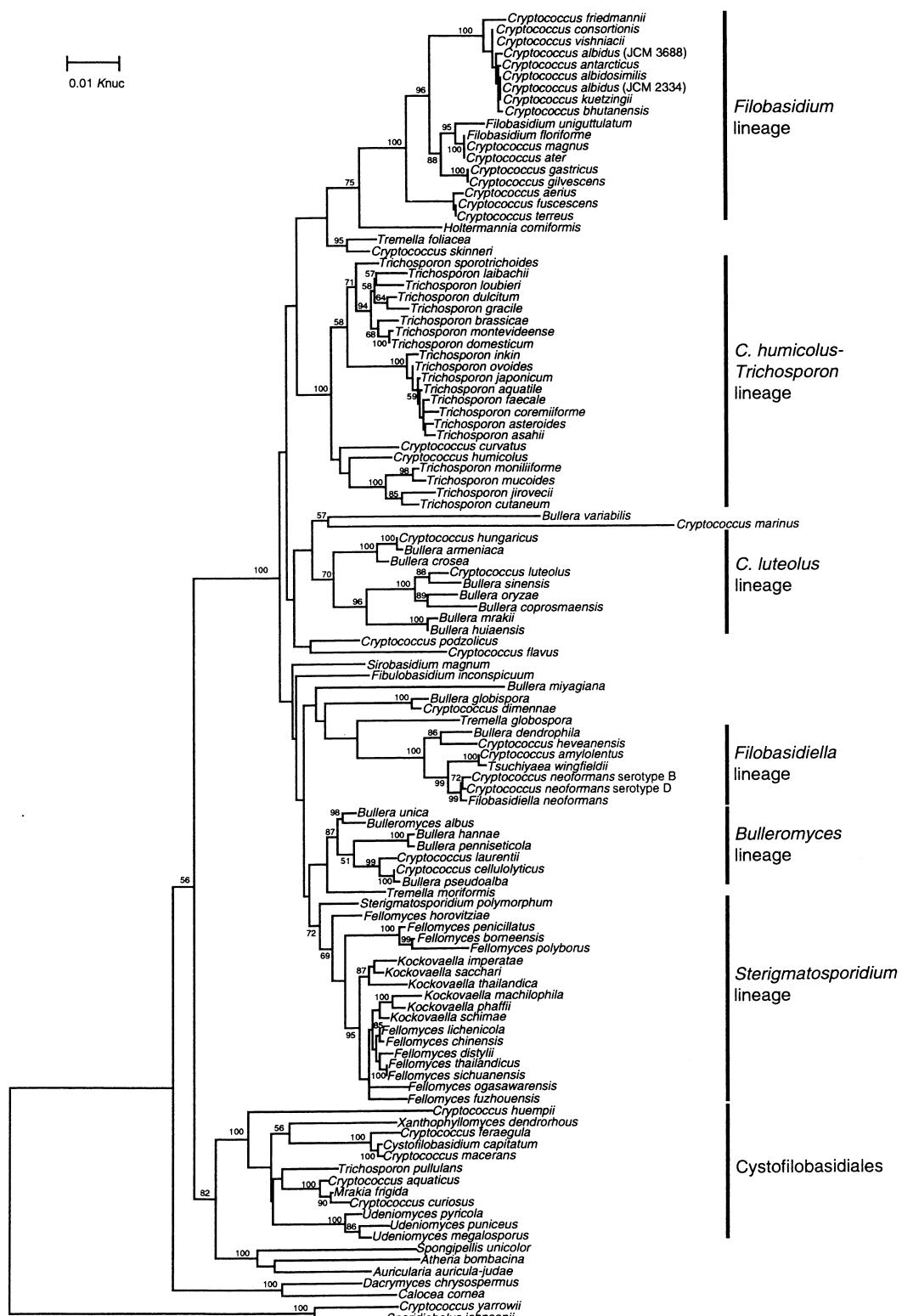


Fig. 1. Neighbor-joining tree of the genus *Cryptococcus* and related taxa based on the 18S rDNA sequences

The evolutionary distance was calculated according to Kimura (1980). The numerals represent the percentages from 100 replicate bootstrap samplings (a frequency of less than 50 % is not shown).

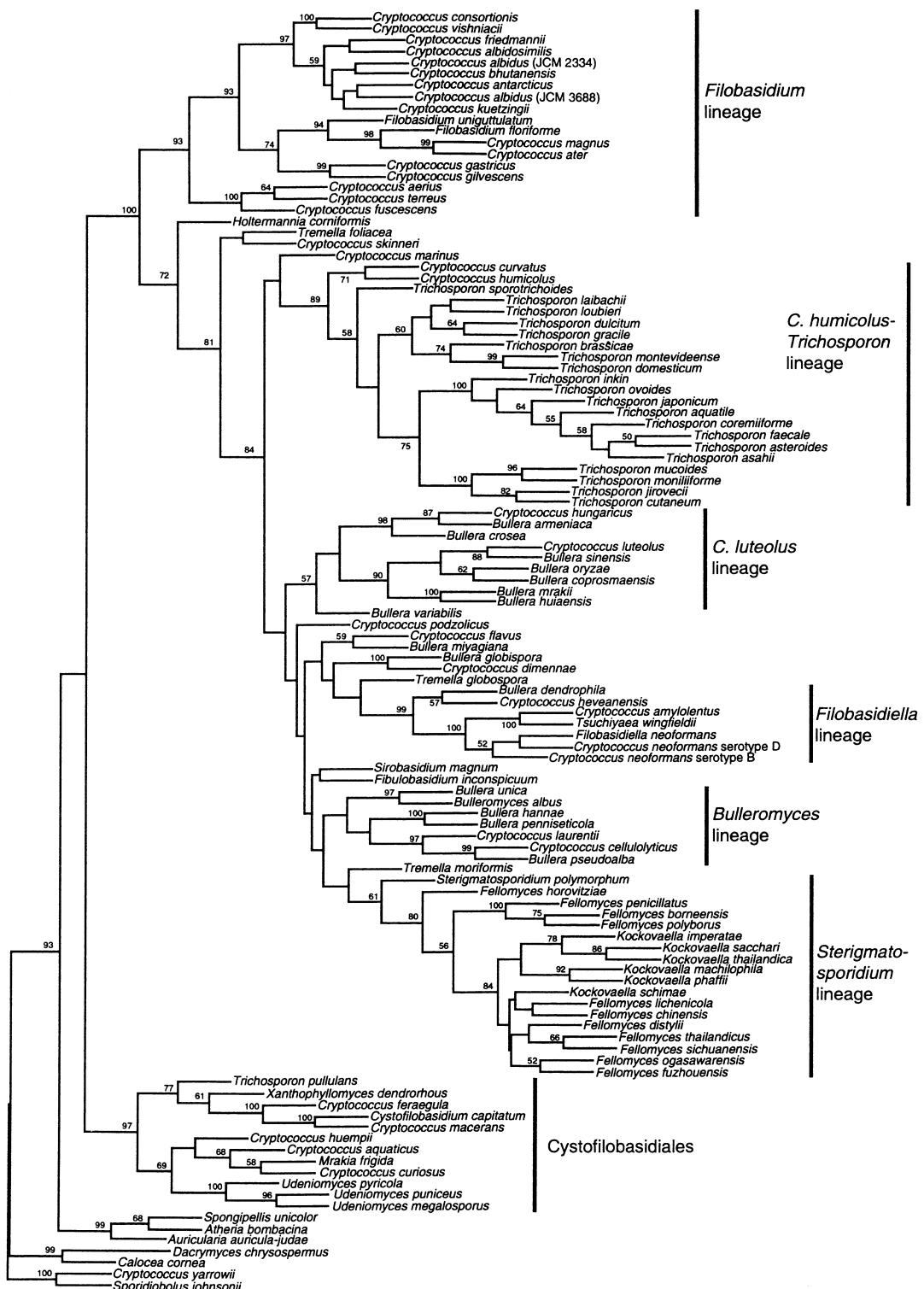


Fig. 2. Maximum parsimony tree of the genus *Cryptococcus* and related taxa based on the 18S rDNA sequences

The numerals represent the percentages from 100 replicate bootstrap samplings (a frequency of less than 50 % is not shown).

Table 3. Grouping of *Cryptococcus* species based on 18S rDNA sequences

Group	Relative to :	Group	Relative to :
1. <i>Filobasidium</i> lineage		4. <i>Bulleromyces</i> lineage	
<i>C. aerius</i> <i>C. albidosimilis</i> <i>C. albidus</i> <i>C. antarcticus</i> <i>C. ater</i> <i>C. bhutanensis</i> <i>C. consortiumis</i> <i>C. friedmannii</i> <i>C. fuscescens</i> <i>C. gastricus</i> <i>C. gilvescens</i> <i>C. kuetzingii</i> <i>C. magnus</i> <i>C. terreus</i> <i>C. vishniacii</i>	⇒ [<i>Filobasidium floriforme</i> <i>Filobasidium uniguttulatus</i>]	<i>C. cellulolyticus</i> <i>C. laurentii</i>	⇒ [<i>Bulleromyces albus</i> <i>Bullera hannaë</i> <i>Bullera penniseticola</i> <i>Bullera pseudoalba</i> <i>Bullera unica</i>]
2. <i>Filobasidiella</i> lineage		5. <i>C. luteolus</i> lineage	
<i>C. amylorentus</i> <i>C. heveanensis</i>	⇒ [<i>Bullera dendrophila</i> <i>Filobasidiella neoformans</i> <i>Tsuchiyaea wingfieldii</i>]	<i>C. hungaricus</i> <i>C. luteolus</i>	⇒ [<i>Bullera armeniaca</i> <i>Bullera coprosmaensis</i> <i>Bullera crocea</i> <i>Bullera huiaensis</i> <i>Bullera mrankii</i> <i>Bullera oryzae</i> <i>Bullera sinensis</i>]
3. <i>C. humigatus</i> - <i>Trichosporon</i> lineage		Others	
<i>C. curvatus</i> <i>C. humiculus</i>	⇒ [<i>Trichosporon</i> species]	<i>C. skinneri</i> <i>C. podzolicus</i> <i>C. dimennae</i> <i>C. flavus</i> <i>C. marinus</i>	⇒ [<i>Tremella foliacea</i> ? ⇒ <i>Bullera grobispora</i> ? ?]
Cystofilobasidiales			
<i>C. aquaticus</i> <i>C. feraegula</i> <i>C. huempii</i> <i>C. macerans</i>	⇒ [<i>Cystofilobasidium capitatum</i> <i>Mrakia frigida</i> <i>Xanthophyllomyces dendrorhous</i> <i>Trichosporon pullulans</i> <i>Udeniomyces</i> species]		
Urediniomycetes			
<i>C. yarrowii</i>			

***Bulleromyces* lineage**

Two *Cryptococcus* species, *C. cellulolyticus* and *C. laurentii*, were located at the *Bulleromyces* lineage with *Bulleromyces albus*, *Bullera hannaë*, *B. penniseticola*, *B. pseudoalba* and *B. unica*. Kurtzman (10) reported the mating types of *C. laurentii* isolated from wheat and corn, but did not propose a teleomorph because he could not observe the sexual state of this species. He also reported that these isolates did not mate with other strains of *C. laurentii*. Vancanneyt et al.(28) indicated the heterogeneity of this species based on G+C mol % and electrophoretic patterns of whole cell protein. It seems important for understanding the phylogeny of this lineage to resolve the heterogeneity of the *C. laurentii*.

ogeneity of this species based on G+C mol % and electrophoretic patterns of whole cell protein. It seems important for understanding the phylogeny of this lineage to resolve the heterogeneity of the *C. laurentii*.

***Filobasidiella* lineage**

The *Filobasidiella* lineage consisted of five species : *Bullera dendrophila*, *Cryptococcus amylorentus*, *C. heveanensis*, *Filobasidiella neoformans* (anamorph *C. neoformans*) and *Tsuchiyaea*

wingfieldii. The genus *Filobasidiella* is included in the *Filobasidiaceae* in Filobasidiales based on the morphology of the basidia in the present taxonomy of basidiomycetous yeasts. However, this genus showed a phylogenetically close relationship with Tremellales in the study. The same results were reported by Guého et al.(7) and Fell et al.(1, 3).

C. amylorentus and *T. wingfieldii* constituted a cluster with *F. neoformans* at a high bootstrap level. Q-10 was contained as a major ubiquinone in *F. neoformans* (22), however, Q-9 was included in *C. amylorentus* and *T. wingfieldii* (28, 30). From these viewpoints, the *Filobasidiella* lineage seemed to be heterogeneous, although only a small number of members is recognized in this lineage.

Filobasidium lineage

The *Filobasidium* lineage consisted of *Cryptococcus albidus*, *C. terreus*, *C. vishniacii*, *Filobasidium floriforme* and *F. uniguttulatum*, and related species (Fig. 1). The *Filobasidium* lineage contained three main branches, the *C. albidus*-*C. vishniacii* branch, the *Filobasidium* related species branch and the *C. terreus* branch. The members of each branch were shown to be extremely closely related based on 18S rDNA sequences as shown in Table 4. Consequently, the sequences of ITS regions were compared among members of the *Filobasidium* lineage (Table 5). The identical sequences of ITS1 and the high sequence similarity of ITS2 among three strains of *C. terreus*, JCM 10453 (type strain), JCM 8975 (formerly *C. elinovii*) and JCM 9493 (formerly *C. himalayaensis*), indicated the conspecificity of these strains (20). In the case of *C. vishniacii*, the sequences of ITS regions were almost identical, and only one base difference (222 bp compared) was detected in the ITS2 between *C. vishniacii* JCM 9430 (formerly *C. socialis*) and other strains of *C. vishniacii* including type strain JCM 10482. Fell and Statzell-Tallman (4) described the synonymy of *C. elinovii* and *C. himalayaensis* to *C. terreus*, and *C. vishniacii* strains used in this study on the basis of the sequences of D1/D2 of 26S rDNA. They also indicated the close relationships between the following three pairs : *C. albidus* and *C. kuetzingii*, *C. antarcticus* and *C. bhutanensis*, and *C. ater* and *C. magnus*. Our data support their reidentification of respective species, *C. terreus* and *C. vishniacii*. Moreover, we found that three pairs, *C. consorts* and *C. vishniacii*, *C.*

gastricus and *C. gilvescens*, and *C. fuscens* and *C. terrues*, showed extremely close relationships.

C. albidus JCM 3688 was formerly treated as *C. albidus* var. *diffluens*. Sugita et al.(21) reported the separation of three varieties of *C. albidus*, *C. albidus* var. *aerius*, *C. albidus* var. *albidus*, and *C. albidus* var. *diffluens*, based on the DNA relatedness value among them. In "The Yeasts, A Taxonomic Study", 4th ed., *C. albidus* var. *aerius* is recognized as a distinct species, *C. aerius*, but *C. albidus* var. *diffluens* is treated as a synonym of *C. albidus* (4). Rodrigues de Miranda (16) pointed out the difficulty of distinguishing these three varieties by their physiological and biochemical properties, however, the comparison of the sequence in the ITS region clearly showed the distinction between them. Furthermore, the identical sequences of ITS regions of *C. albidus* JCM 3688 and *C. albidosimilis* indicated the conspecificity of these species (Table 5). As described by Vancanneyt et al.(28), *C. albidus* is a heterogeneous species, and further study will be required for the reclassification of it and related species.

C. humiculus-Trichosporon lineage

In the *C. humiculus-Trichosporon* lineage, *C. cruvatus* and *C. humiculus* were clustered with *Trichosporon* species, except for *T. pullulans*, with a high bootstrap value (Figs. 1 and 2). However, the phylogenetic position of *C. curvatus* and *C. humiculus* to *Trichosporon* species was not reliable as shown in the neighbor-joining tree (Fig. 1) and the maximum parsimony tree (Fig. 2). These topologies were similar to those in other reports (1, 7). *C. cruvatus* and *C. humiculus* are distinguished from *Trichosporon* species by their morphological characteristics. The intraspecific heterogeneity of *C. humiculus* was assumed by its possessing both Q-9 and Q-10 strains as a major ubiquinone (28). Sugita and Nakase (18) reported that phylogenetic groups on the genus *Trichosporon* corresponded to the major ubiquinone types, Q-9 and Q-10. We believe that intraspecific investigation of *C. cruvatus* and *C. humiculus* is necessary to determine the relationship among these species and *Trichosporon* species.

C. luteolus lineage

The *C. luteolus* lineage contained *Bullera armeniaca*, *B. crocea*, *B. coprosmaensis*, *B. huiaensis*,

Table 4. Base differences of 18S rDNA sequences among species of the *Filobasidium* lineage

Species	Strain	Number of base differences*																			
		JCM 2334	JCM 8843	JCM 9037	JCM 9424	JCM 9426	JCM 10482	JCM 9422	JCM 9423	JCM 9428	JCM 9047	JCM 9430	JCM 9431	JCM 3692	JCM 9884	JCM 10451	JCM 3685	JCM 9427	JCM 9493	JCM 10453	JCM 10481
<i>C. albidus</i>	JCM 2334	-																			
<i>C. albidosimilis</i>	JCM 8843	0	-																		
<i>C. kuetzingii</i>	JCM 9037	0	0	-																	
<i>C. bhutanensis</i>	JCM 9424	1	1	1	-																
<i>C. antarcticus</i>	JCM 8844	1	1	1	2	-															
<i>C. albidus</i> (= <i>C. albidus</i> var. <i>diffluens</i>)	JCM 3688	2	2	2	3	3	-														
<i>C. friedmannii</i>	JCM 9426	3	3	3	4	4	5	-													
<i>C. vishniacii</i>	JCM 10482	1	1	1	2	2	3	4	-												
<i>C. vishniacii</i> (= <i>C. asgardensis</i>)	JCM 9422	1	1	1	2	2	3	4	0	-											
<i>C. vishniacii</i> (= <i>C. baldrensis</i>)	JCM 9423	1	1	1	2	2	3	4	0	0	-										
<i>C. vishniacii</i> (= <i>C. hempflingii</i>)	JCM 9428	1	1	1	2	2	3	4	0	0	0	-									
<i>C. vishniacii</i> (= <i>C. lupi</i>)	JCM 9047	1	1	1	2	2	3	4	0	0	0	0	-								
<i>C. vishniacii</i> (= <i>C. socialis</i>)	JCM 9430	1	1	1	2	2	3	4	0	0	0	0	0	-							
<i>C. vishniacii</i> (= <i>C. tyrolensis</i>)	JCM 9431	1	1	1	2	2	3	4	0	0	0	0	0	-							
<i>C. vishniacii</i> (= <i>C. vishniacii</i> var. <i>asocialis</i>)	JCM 9049	1	1	1	2	2	3	4	0	0	0	0	0	-							
<i>C. consortonis</i>	JCM 9425	1	1	1	2	2	3	4	0	0	0	0	0	0	-						
<i>C. gastricus</i>	JCM 3691	27	27	27	28	26	29	30	26	26	26	26	26	26	26	26	26	26	-		
<i>C. gastricus</i>	JCM 3692	27	27	27	28	26	29	30	26	26	26	26	26	26	26	26	26	26	0	-	
<i>C. gilvescens</i>	JCM 9884	27	27	27	28	26	29	30	26	26	26	26	26	26	26	26	26	26	0	0	-
<i>C. ater</i>	JCM 10451	27	27	27	28	26	29	30	26	26	26	26	26	26	26	26	26	26	16	16	-
<i>C. magnus</i>	JCM 9038	27	27	27	28	26	29	30	26	26	26	26	26	26	26	26	26	16	16	0	-
<i>F. uniguttulatum</i>	JCM 3685	32	32	32	33	31	34	35	31	31	31	31	31	31	31	31	17	17	17	11	11
<i>C. fuscescens</i>	JCM 9427	33	33	33	34	34	35	36	32	32	32	32	32	32	32	32	32	32	33	-	
<i>C. terreus</i>	JCM 10453	33	33	33	34	34	35	36	32	32	32	32	32	32	32	32	32	32	33	0	-
<i>C. terreus</i> (= <i>C. elinovii</i>)	JCM 8975	33	33	33	34	34	35	36	32	32	32	32	32	32	32	32	32	32	33	0	0
<i>C. terreus</i> (= <i>C. himalayaensis</i>)	JCM 9493	33	33	33	34	34	35	36	32	32	32	32	32	32	32	32	32	32	33	0	0
<i>C. aerius</i>	JCM 10481	34	34	34	35	35	36	37	33	33	33	33	33	33	33	33	25	25	33	33	34
																	1	1	1	1	

*, 1781 bases used.

Table 5. The sequence similarity of ITS region among species in the *Filobasidium* lineage^a

Species	Strain	JCM 2334	JCM 9037	JCM 9424	JCM 8844	JCM 3688	JCM 8843	JCM 9425	JCM 10482	JCM 9422	JCM 9423	JCM 9428	JCM 9047	JCM 9431	JCM 9049	JCM 9430	JCM 9426	JCM 3691	JCM 3692	JCM 9884	JCM 10451	JCM 9038	JCM 3685	JCM 9427	JCM 10453	JCM 8975	JCM 9493	JCM 10481		
<i>C. albidus</i>	JCM 2334	-	99.4	91.7	91	94.8	94.2	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1		
<i>C. kuetzingii</i>	JCM 9037	99.5	-	92.4	91.7	95.5	94.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	74.0		
<i>C. bhutanensis</i>	JCM 9424	96.9	97.3	-	97.5	91.7	92.4	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	75.2		
<i>C. antarcticus</i>	JCM 8844	96.0	96.4	98.7	-	91.1	91.7	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	75.8			
<i>C. albidus</i> (= <i>C. albidus</i> var. <i>diffluens</i>)	JCM 3688	97.3	98.2	96.9	97.3	-	100	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	75.2		
<i>C. albidosimilis</i>	JCM 8843	97.3	94.8	92.4	91.7	100	-	94.2	94.2	94.2	94.2	94.2	94.2	94.2	94.2	94.2	94.2	94.2	94.2	94.2	94.2	94.2	94.2	94.2	94.2	94.2	74.5			
<i>C. consortium</i>	JCM 9425	98.6	98.2	96.9	96.9	98.6	98.6	-	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	75.2			
<i>C. vishniacii</i>	JCM 10482	98.6	98.2	96.9	96.9	98.6	98.6	100	-	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	75.2			
<i>C. vishniacii</i> (= <i>C. asgardenensis</i>)	JCM 9422	98.6	98.2	96.9	96.9	98.6	98.6	100	100	-	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	75.2			
<i>C. vishniacii</i> (= <i>C. baldrensis</i>)	JCM 9423	98.6	98.2	96.9	96.9	98.6	98.6	100	100	100	-	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	75.2			
<i>C. vishniacii</i> (= <i>C. hemphlingii</i>)	JCM 9428	98.6	98.2	96.9	96.9	98.6	98.6	100	100	100	100	-	100	100	100	100	100	100	100	100	100	100	100	100	100	100	75.2			
<i>C. vishniacii</i> (= <i>C. lupi</i>)	JCM 9047	98.6	98.2	96.9	96.9	98.6	98.6	100	100	100	100	100	-	100	100	100	100	100	100	100	100	100	100	100	100	100	75.2			
<i>C. vishniacii</i> (= <i>C. tyrolensis</i>)	JCM 9431	98.6	98.2	96.9	96.9	98.6	98.6	100	100	100	100	100	100	-	100	100	100	100	100	100	100	100	100	100	100	100	75.2			
<i>C. vishniacii</i> (= <i>C. vishniacii</i> var. <i>asociialis</i>)	JCM 9049	98.6	98.2	96.9	96.9	98.6	98.6	100	100	100	100	100	100	100	-	100	100	100	100	100	100	100	100	100	100	100	75.2			
<i>C. vishniacii</i> (= <i>C. socialis</i>)	JCM 9430	98.2	97.7	96.4	96.4	98.2	98.2	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	-	75.2										
<i>C. friedmannii</i>	JCM 9426	93.8	94.2	96.4	97.3	95.1	95.1	94.6	94.6	94.6	94.6	94.6	94.6	94.6	94.6	94.6	94.6	94.6	94.2	-										
<i>C. gastricus</i>	JCM 3691																			-	99.3	100								
<i>C. gastricus</i>	JCM 3692																			99.6	-	99.3								
<i>C. gilvescens</i>	JCM 9884																			98.8	99.2	-								
<i>C. ater</i>	JCM 10451																						-	100	74.5					
<i>C. magnus</i>	JCM 9038																			100	-	74.5								
<i>F. uniguttulatum</i>	JCM 3685																			86.3	86.3	-								
<i>C. fuscescens</i>	JCM 9427																						-	98.8	98.8	98.8	98.8	93.4		
<i>C. terreus</i>	JCM 10453																						99.6	-	100	100	93.4			
<i>C. terreus</i> (= <i>C. elinovii</i>)	JCM 8975																						99.6	99.1	-	100	93.4			
<i>C. terreus</i> (= <i>C. himalayaensis</i>)	JCM 9493																						100	99.6	99.6	-	93.4			
<i>C. aerius</i>	JCM 10481																						99.1	98.7	98.7	99.1	-			

^a, Upper right triangle shows ITS 1 similarity, and lower left triangle shows ITS 2 similarity.

B. mrakii, *B. oryzae*, *B. sinensis*, *Cryptococcus hungaricus* and *C. luteolus*. *Bullera variabilis* showed a relationship to this lineage but its phylogenetic position was not stable depending on the methods of the analyses (Figs. 1 and 2). The orange colored species, *C. hungaricus*, showed a close relationship with *B. armeniaca* and *B. crocea*, whose colonies were also orange. *C. luteolus* constituted another cluster with *B. coprosmaensis*, *B. huiensis*, *B. mrakii*, *B. oryzae* and *B. sinensis*. The heterogeneity of *B. variabilis* and *C. luteolus* were reported by Nakase and Suzuki (14) and Vancanneyt et al. (28), respectively. The *C. luteolus* lineage is probably divided into several phylogenetic groups after intraspecific study of *B. variabilis* and *C. luteolus*.

Sterigmatosporidium lineage

The *Sterigmatosporidium* lineage was constituted of the genera *Fellomyces*, *Kockovaella* and *Sterigmatosporidium*, and did not include *Cryptococcus* species. This cluster was characterized by Q-10 as a major ubiquinone and stalked conidia-producing activity. This indicated that stalked conidia-producing activity seemed to reflect the phylogenetic relationship among strains. On the other hand, ballistoconidia-forming activity distributed in the four lineages (the genera *Bulleromyces*, *Bullera*, *Kockovaella* and *Udeniomyces*, appeared in the *Bulleromyces* lineage, the *Filobasidiella* lineage, the *C. luteolus* lineage and Cystofilobasidiales) did not correspond to the phylogeny. Hibbett et al. (8) reported that the same phenomenon was observed in the case of Agaricales and Gasteromycetes. Stalked conidia-forming activity and ballistoconidia-forming activity are now employed as taxonomic criteria of a generic level in basidiomycetous yeasts. The taxonomic significance of ballistoconidia-forming activity should be reexamined using more phylogenetic and morphological approaches. Stalked conidia-forming activity seemed to be phylogenetically important characteristics as shown in the *Sterigmatosporidium* lineage. One species, *Tsuchiyaea wingfieldii*, which contained Q-9 as a major unquinone and produced stalked conidia, made a cluster with *C. amylorentus* in the *Filobasidiella* lineage. The mode of conidiogenesis of this species was reported to be different from that of *Fellomyces* (27). Therefore, further phylogenetic and morphological studies are

required to confirm the relationship between stalked conidia-producing activity and phylogeny.

Cryptococcus species, except for those in the *Filobasidiella* lineage, would be reclassified to other genera according to phylogenetic relationship among hymenomycetous species, as the neotype species of the genus is *C. neoformans*, anamorph of *F. neoformans*. However, it is impossible to distinguish each lineage based on the present taxonomic criteria in basidiomycetous yeasts. The *Sterigmatosporidium* lineage seemed to be distinct in this respect. The phenotypic properties that characterize each lineage must be sought and the taxonomic significance of morphology of the basidia, which is regarded as one of the most important criteria, should be reexamined as indicated by Fell et al. (3).

In conclusion, the phylogenetic heterogeneity of the genus *Cryptococcus* was shown in this study. *Cryptococcus* species, except for *C. yarrowii*, will probably be divided into several genera in Filobasidiales, Tremellales and Cystofilobasidiales. Further investigation focused on the heterogeneous species, *C. albidus*, *C. humicolus*, *C. luteolus* and *C. laurentii*, is necessary to understand the phylogenetic relationship among them and related species. *C. yarrowii*, which shows the urediniomycetous affinity, should be transferred to the genus *Rhodotorula*. Some extremely closely related species revealed from the sequence analyses of ITS regions, such as *C. albidus* and *C. kuetzingii*, will be reclassified after DNA-DNA reassociation experiments.

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18S rDNA および ITS 領域の塩基配列に基づく *Cryptococcus* 属酵母
および関連菌類の系統関係

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Cryptococcus 属の基準株 33 株, その他数株の関連菌株の 18S rDNA 塩基配列を決定し, 関連菌株とあわせて, 近隣結合法および最大節約法を用いて系統樹を作成した。また, 18S rDNA の解析により, 系統的に近縁であると推定された種について, internal transcribed spacer region(ITS 領域)の配列を決定し比較を行った。*Cryptococcus* 属の種は, *C. yarrowii* を除いて大きく 2 つのグループに分かれた。*C. aquaticus*, *C. curiosus* (= *Mrakia frigida*), *C. feraegula*, *C. hempfii*, および *C. macerans* の 5 種は Cystofilobasidiales と近縁であった。23 種は Filobasidiales および Tremellales のグループのうち, 5 つの主な系統枝 (*Bullelomyces* lineage, *Filobasidiella* lineage, *Filobasidium* lineage, *C. humicolus-Trichosporon* lineage, および *C. luteolus* lineage) に, ほかの属の種と混じって位置していた。残りの 5 種は Filobasidiales/Tremellales の系統枝に位置したが, 上記 5 つの系統枝には含まれず, アラインメントや系統樹作成法により位置が異なっていた。ITS 領域の塩基配列の解析により, 以下の数組の種が, 非常に系統的に近縁であることが示された: *C. albidus* JCM 2334 (*C. albidus* の基準株) および *C. kuetzingii*, *C. albidus* JCM 3688 (*C. albidus* var. *diffluence* の基準株) および *C. albitosimilis*, *C. antarcticus* および *C. bhutanensis*, *C. ater* および *C. magnus*, *C. consortions* および *C. vishniacii*, *C. gastricus* および *C. gilvescens*, および *C. fuscensens* および *C. terreus*. *C. yarrowii* はサビキン綱に位置した。