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Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis

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The molecular systematics of 337 strains of basidiomycetous yeasts and yeast-like fungi, representing 230 species in 18 anamorphic and 24 teleomorphic genera, was determined by sequence analysis of the D1/D2 region of the large-subunit rDNA. The data were compared with published sequences of other basidiomycetous fungi. The results demonstrated that the yeast species and genera are phylogenetically distributed among the *Microbotryum*, *Sporidiobolus*, *Agaricostilbum* and *Erythrobasidium* clades of the Urediniomycetes; the Tremellales, Trichosporonales ord. nov., Filobasidiales and Cystofilobasidiales clades of the Hymenomycetes; and the Ustilaginales, Microstromatales and Malasseziales clades of the Ustilaginomycetes. Genera such as *Bensingtonia*, *Cryptococcus*, *Rhodotorula* and *Sporobolomyces* are polyphyletic, i.e. they occur in two or more clades. In contrast, other genera, e.g. *Bullera*, *Cystofilobasidium*, *Fellomyces*, *Filobasidiella*, *Filobasidium*, *Kondoa*, *Kurtzmanomyces*, *Leucosporidium*, *Rhodosporidium*, *Sporidiobolus* and *Udeniomyces*, are monophyletic. The majority of the species can be identified using D1/D2 analyses, although the internal transcribed spacer region is required to distinguish closely related species. The intergenic spacer region is recommended for additional differentiation of species and strains.

Keywords: yeasts, Urediniomycetes, Hymenomycetes, Ustilaginomycetes, Trichosporonales ord. nov.

INTRODUCTION

The basidiomycetous yeasts, as currently recognized, are distributed among the three classes of the Basidiomycota: Ustilaginomycetes, Urediniomycetes and Hymenomycetes. These yeasts have considerable economic, agricultural and medical importance and estimates indicate that the number of known yeasts may represent about 1% of the species that exist in nature. There is an increased interest in discovering these species for economic exploitation and there is a need to understand their biodiversity and ecological roles. Identification and phylogenetic placement of the basidiomycetous yeasts are not always easy to accomplish, partly because of their polyphyletic nature. The unifying characteristic of these fungi is a predominant unicellular growth phase. Separation of yeasts into the three classes of fungi is based on septal morphology, cell wall composition and rDNA analysis. Generic diagnoses are directed to sexual and vegetative biology, in addition to physiological tests such as growth on inositol or D-glucuronic acid and formation of extracellular starch-like compounds. Species are usually differentiated by physiological attributes, particularly the utilization of carbon and nitrogen sources, and by measurement of DNA reassociations between closely related species. By the very nature of these tests, identifications can be slow, difficult and expensive to perform; results of physiological and morphological tests often demonstrate considerable variability within and between species. Consequently, there is an urgent need for diagnostic tools to provide rapid and accurate

Abbreviations: IGS, intergenic spacer; ITS, internal transcribed spacer; LrDNA, large-subunit rDNA.

Table 1. Hymenomycetous yeasts examined in the D1/D2 and internal transcribed spacer rDNA regions

Strain	Strain no.*	GenBank accession no.	
		D1/D2	ITS†
Apiotrichum porosum	CBS 2040^T	AF189833	
Bullera armeniaca	CBS 7091^T	AF189883	
Bullera crocea	CBS 6714^T	AF075508	
Bullera dendrophila	CBS 6074^T	AF189870	
Bullera derxii	CBS 7225^T	AF189857	
Bullera globispora	CBS 6981^T	AF075509	
Bullera miyagiana	CBS 7526^T	AF189858	
Bullera oryzae	CBS 7194^T	AF075511	
Bullera pseudoalba	CBS 7227^T	AF075504	
Bullera sinensis	CBS 7238^T	AF189884	
Bullera unica	CBS 8290^T	AF075524	
Bullera variabilis	CBS 7347^T	AF189855	
Bulleromyces albus	CBS 501^T	AF075500	
Cryptococcus adeliensis	CBS 8351^T	AF137603	AF145328
Cryptococcus aerius	CBS 155^T	AF075486	AF145324
Cryptococcus albidosimilis	CBS 7711^T	AF137601	AF145325
Cryptococcus albidosimilis	ATCC 34633	AF137606	AF145331
Cryptococcus albidus	CBS 142^T	AF075474	AF145321
Cryptococcus albidus	IGC 2426	AF181514	
Cryptococcus albidus	IGC 4789	AF181531	
Cryptococcus albidus	IGC 4963	AF181509	
Cryptococcus albidus	IGC 4990	AF181511	
Cryptococcus genitalis‡	CBS 5592^T	AF181538	
Torulopsis nadaensis‡	CBS 969^T	AF181516	
Torulopsis rotundata‡	CBS 945^T	AF181517	
Cryptococcus albidus var. *ovalis*	CBS 5810^T	AF137605	AF145329
Cryptococcus amylolentus	CBS 6039^T	AF105391	
Cryptococcus antarcticus	CBS 7687^T	AF075488	AF145326
Cryptococcus aquaticus	CBS 5443^T	AF075470	
Cryptococcus bhutanensis	CBS 6294^T	AF137599	AF145317
Cryptococcus cellulolyticus	CBS 8294^T	AF075525	
Cryptococcus curvatus	CBS 570^T	AF189834	
Cryptococcus dimennae	CBS 5770^T	AF075489	
Cryptococcus diffluens	CBS 160^T	AF075502	AF145330
Cryptococcus diffluens var. *uruguaiensis*‡	CBS 6436^T	AF181543	
Torulopsis albida var. *japonica*‡	CBS 926^T	AF181542	
Cryptococcus elinovii	CBS 7051^T	AF137604	AF145318
Cryptococcus flavus	CBS 331^T	AF075497	
Cryptococcus friedmannii	CBS 7160^T	AF075478	AF145322
Cryptococcus fuscescens	CBS 7189^T	AF075472	AF145319
Cryptococcus gastricus	CBS 2288^T	AF137600	
Cryptococcus gastricus	CBS 1927	AF181501	
Cryptococcus gilvescens	CBS 7525^T	AF181547	
Cryptococcus heveanensis	CBS 569^T	AF075467	
Cryptococcus himalayensis	CBS 6293^T	AF181502	
Cryptococcus huempii	CBS 8186^T	AF189844	
Cryptococcus humicolus	CBS 571^T	AF189836	
Cryptococcus humicolus	CBS 8354	AF189851	
Cryptococcus humicolus	CBS 8371	AF189854	
Cryptococcus hungaricus	CBS 4214^T	AF075503	
Cryptococcus kuetzingii	CBS 1926^T	AF137602	AF145327

Table 1 (*cont.*)

Strain	Strain no.*	GenBank accession no.	
		D1/D2	ITS†
Cryptococcus kuetzingii	CBS 922^T	AF181504	
Cryptococcus laurentii	CBS 139^T	AF075469	
Cryptococcus luteolus	CBS 943^T	AF075482	
Cryptococcus macerans	CBS 2206^T	AF189848	
Cryptococcus macerans	CBS 2425	AF075477	
Cryptococcus magnus	CBS 140^T	AF181851	AF190008
Cryptococcus magnus	CBS 8361	AF189852	
Cryptococcus magnus	CBS 8362	AF189853	
Cryptococcus magnus	CBS 8394	AF189872	
Cryptococcus magnus	IGC 4556	AF181528	
Cryptococcus magnus	IGC 4563	AF181529	
Cryptococcus magnus	IGC 4989	AF181510	
Cryptococcus magnus	IGC 5260	AF181532	
Cryptococcus magnus	IGC 5267	AF181536	
Cryptococcus ater‡	CBS 4685^T	AF181505	AF190009
Cryptococcus marinus	CBS 5235^T	AF189846	
Cryptococcus podzolicus	CBS 6819^T	AF075481	
Cryptococcus skinneri	CBS 5029^T	AF189835	
Cryptococcus terreus	CBS 1895^T	AF075479	
Cryptococcus terricolus	CBS 4517^T	AF181520	
Cryptococcus terricolus	CBS 6435	AF181545	
Cryptococcus vishniacii	CBS 7110^T	AF075473	AF145320
Cryptococcus asgardensis‡	CBS 8141^T	AF189839	
Cryptococcus baldrensis‡	CBS 8142^T	AF189840	
Cryptococcus consortionis‡	A801-3aY92/20^T	AF189880	
Cryptococcus hempflingii‡	CBS 8143^T	AF189841	
Cryptococcus lupi‡	CBS 8100^T	AF189860	
Cryptococcus socialis‡	CBS 7158^T	AF181503	
Cryptococcus vishniacii var. *asocialis*‡	CBS 8146^T	AF189838	
Cryptococcus wrightensis‡	CBS 8145^T	AF189837	
Cystofilobasidium bisporidii	CBS 6346^T	AF189832	
Cystofilobasidium bisporidii	CBS 6347	AF075464	
Cystofilobasidium capitatum	CBS 6358^T	AF075465	AF139627
Cystofilobasidium lari-marini‡	CBS 7420^T	AF075466	
Cystofilobasidium feraegula	CBS 7201	AF075487	
Cystofilobasidium infirmo-miniatum	CBS 323^T	AF075505	
Fellomyces borneensis	CBS 8282^T	AF189877	
Fellomyces chinensis	CBS 8278^T	AF189878	
Fellomyces fuzhouensis	CBS 6133	AF075506	
Fellomyces horovitziae	CBS 7515^T	AF189856	
Fellomyces penicillatus	CBS 5492^T	AF177405	
Fellomyces polyborus	CBS 6072^T	AF189859	
Fellomyces sichuanensis	CBS 8318^T	AF189879	
Filobasidiella neoformans var. *neoformans*	CBS 132^T	AF075484	
Filobasidiella neoformans var. *neoformans*	CBS 882	AF189845	
Filobasidiella neoformans var. *bacillispora*	CBS 6289^T	AF075526	
Filobasidium capsuligenum	CBS 4736	AF075501	
Filobasidium capsuligenum	CBS 6219	AF181506	
Filobasidium elegans	CBS 7640	AF181548	AF190006
Filobasidium floriforme	CBS 6241^T	AF075498	AF190007

Table 1 (*cont.*)

Strain	Strain no.*	GenBank accession no.	
		D1/D2	ITS†
Filobasidium globisporum	CBS 7642	AF075495	
Filobasidium uniguttulatum	CBS 1730^T	AF075468	
Filobasidium uniguttulatum	CBS 1727	AF181500	
Holtermannia corniformis	CBS 6979	AF189843	
Kockovaella imperatae	CBS 7554^T	AF189862	
Kockovaella thailandica	CBS 7552^T	AF075516	
Mrakia frigida	CBS 5270^T	AF075463	AF144483
Mrakia nivalis‡	CBS 5266^T	AF189849	AF144484
Cryptococcus curiosus‡	CBS 5688^T	AF189847	AF144482
Mrakia gelida	CBS 5272^T	AF189831	AF144485
Mrakia stokesii‡	CBS 5917^T	AF189830	AF144486
Naganishia globosa	CBS 5106^T	AF181539	
Cryptococcus sp.	IGC 5257	AF181512	
Hansenula amylofaciens‡	CBS 1975^T	AF181540	
Phaffia rhodozyma	CBS 5905^T	AF189871	
Sirobasidium magnum	CBS 6803	AF075475	
Sirobasidium intermedium	CBS 7805	AF075492	
Sterigmatosporidium polymorphum	CBS 8088^T	AF075480	
Torulopsis liquefaciens	CBS 968^T	AF181515	
Cryptococcus sp.	IGC 2406	AF181513	
Cryptococcus sp.	IGC 2934	AF181518	
Torulopsis pseudoaeria	CBS 4192^T	AF181544	
Cryptococcus sp.	IGC 4643	AF181522	
Cryptococcus sp.	IGC 5259	AF181525	
Tremella aurantia	CBS 6965	AF189842	
Tremella brasiliensis	CBS 6966	AF189864	
Tremella cinnabarina	CBS 8234	AF189866	
Tremella coalescens	CBS 6967	AF189865	
Tremella encephala	CBS 6968	AF189867	
Tremella foliacea	CBS 6969	AF189868	
Tremella fuciformis	CBS 6970	AF075476	
Tremella globispora	CBS 6972	AF189869	
Tremella mesenterica	CBS 6973	AF075518	
Tremella moriformis	CBS 7810	AF075493	
Trichosporon aquatile	CBS 5973^T	AF075520	
Trichosporon asahii	CBS 2479^T	AF105393	
Trichosporon asahii	CBS 8640	AF189881	
Trichosporon asahii	CBS 7137	AF189882	
Trichosporon asahii	CBS 8520	AF189876	
Trichosporon asteroides	CBS 2481^T	AF075513	
Trichosporon brassicae	CBS 6382^T	AF075521	
Trichosporon cutaneum	CBS 2466^T	AF075483	
Trichosporon coremiiforme	CBS 2482^T	AF139983	
Trichosporon coremiiforme	CBS 2478	AF189863	
Trichosporon domesticum	CBS 8280^T	AF075512	
Trichosporon domesticum	CBS 8111	AF189874	
Trichosporon dulcitum	CBS 8257^T	AF075517	
Trichosporon faecale	CBS 4828^T	AF105395	
Trichosporon gracile	CBS 8189^T	AF105399	
Trichosporon gracile	CBS 8518	AF189875	

Table 1 (*cont.*)

Strain	Strain no.*	GenBank accession no.	
		D1/D2	ITS†
Trichosporon guehoae	CBS 8521^T	AF105401	
Trichosporon inkin	CBS 5585^T	AF105396	
Trichosporon jirovecii	CBS 6864^T	AF105398	
Trichosporon laibachii	CBS 5790^T	AF075514	
Trichosporon loubierii	CBS 7065^T	AF075522	
Trichosporon moniliiforme	CBS 2467^T	AF105392	
Trichosporon moniliiforme	CBS 8400	AF189873	
Trichosporon montevideense	CBS 6721^T	AF105397	
Trichosporon mucoides	CBS 7625^T	AF075515	
Trichosporon multisporum	CBS 2495^A	AF139984	
Trichosporon ovoides	CBS 7556^T	AF075523	
Trichosporon pullulans	CBS 2532^T	AF105394	
Trichosporon pullulans	CBS 2541	AF189861	
Trichosporon sporotrichoides	CBS 8246^T	AF189885	
Trichosporon veenhuisii	CBS 7136^T	AF105400	
Tsuchiyaea wingfieldii	CBS 7118^T	AF177404	
Udeniomyces megalosporus	CBS 7236^T	AF075510	
Udeniomyces puniceus	CBS 5689^T	AF075519	
Udeniomyces pyricola	CBS 6754^T	AF075507	
Xanthophyllomyces dendrorhous	CBS 7918^T	AF075496	

* T, Type strain; A, authentic strain.

† Not all strains were examined in the ITS region.

‡ Species considered to be synonyms of the lead listed species as determined by sequence analysis and examination of classical taxonomic characteristics.

identifications of species and to gather information on phylogenetic relationships.

To overcome these problems, yeast-identification techniques have been directed to molecular methods, such as utilization of species-specific PCR primers (Fell, 1995; Haynes *et al*., 1995; Mannarelli & Kurtzman, 1998; Mitchell *et al*., 1994), analysis of RFLPs (Magee *et al*., 1987), PFGE, randomly amplified polymorphic DNA (Boekhout *et al*., 1997) and single-stranded conformational polymorphisms (Walsh *et al*., 1995). Significant advances in basidiomycete systematics resulted from sequence analysis of the large and small subunits of rRNA and DNA (Boekhout *et al*., 1995; Fell & Kurtzman, 1990; Fell *et al*., 1995; Guého *et al*., 1989, 1993; Nakase *et al*., 1993; Sugiyama & Suh, 1993; Suh & Sugiyama, 1993; Suh & Nakase, 1995; Swann & Taylor, 1995b; Van de Peer *et al*., 1992; Yamada & Kawasaki, 1989; Yamada & Nakagawa, 1992).

One of the long-standing problems has been understanding the phylogenetic relationships between basidiomycetous yeasts and filamentous fungi. Dimorphism (yeast and filamentous states) has been recognized as representing distinct stages in the life-histories of heterobasidiomycetous fungi such as species of *Tremella* and *Filobasidiella*. With the exception of *Xanthophyllomyces*, all of the teleomorphic basidiomycetous yeast genera have a filamentous stage. However, the relationships between the basidiomycetous yeasts and other fungi have been an open question. Ultrastructural and molecular analyses have shown that basidiomycetous yeasts are distributed among the three main phylogenetic lines of the Basidiomycota, namely the Hymenomycetes, Urediniomycetes and Ustilaginomycetes (McLaughlin *et al*., 1995; Swann & Taylor, 1995a, c).

The purpose of the current communication is to examine the distribution of yeasts among these three classes of fungi. This is the first study to examine the D1/D2 region of the large-subunit rDNA (LrDNA) for all known species of basidiomycetous yeasts (*sensu* Kurtzman & Fell, 1998). In addition, this report examines the use of the D1/D2 and internal transcribed spacer (ITS) regions for recognizing species boundaries.

METHODS

The strains that we examined are listed in Tables 1–3. Strains and synonyms with identical nucleotide sequences are shown indented under a species name. For example, seven strains were examined that had sequences identical to those of *Cryptococcus albidus* (IGC 2426, 4789, 4963, 4990) and the synonyms *Cryptococcus genitalis*, *Torulopsis rotundata* and *Torulopsis nadaensis*. Type strains of species and synonyms

Table 2. Urediniomycetous yeasts examined in the D1/D2 and ITS rDNA regions

Strain	Strain no.*	GenBank accession no.	
		D1/D2	ITS†
Agaricostilbum hyphaenes	CBS 7811	AF177406	
Bensingtonia ciliata	CBS 7514^T	AF189887	
Bensingtonia ingoldii	CBS 7424^T	AF189888	
Bensingtonia intermedia	CBS 7226^T	AF189889	
Bensingtonia intermedia	CBS 7281	AF189890	
Bensingtonia miscanthi	CBS 7282^T	AF189891	
Bensingtonia musae	CBS 7965^T	AF189892	
Bensingtonia naganoensis	CBS 7286^T	AF189893	
Bensingtonia phyllada	CBS 7169^T	AF189894	
Bensingtonia subrosea	CBS 7283^T	AF189895	
Bensingtonia yamatoana	CBS 7243^T	AF189896	
Bensingtonia yuccicola	CBS 7331^T	AF189897	
Chionosphaera apobasidialis	CBS 7430^T	AF177407	
Colacogloea peniophorae	IGC 4825	AF189898	
Erythrobasidium hasegawianum	CBS 8253^T	AF189899	
Heterogastridium pycnidioideum	CBS 591.93	AF189900	
Kondoa aeria	CBS 8352^T	AF189901	
Kondoa aeria	CBS 8378	AF189902	
Kondoa malvinella	CBS 6082^T	AF189903	
Kondoa myxariophilla	CBS 8379^T	AF189904	
Kriegeria eriophori	CBS 8387	AF189905	
Kurtzmanomyces insolitus	CBS 8377^T	AF177408	
Kurtzmanomyces nectairei	CBS 6405^T	AF177409	
Kurtzmanomyces tardus	CBS 7421^T	AF177410	
Leucosporidium antarcticum	CBS 5942^T	AF189906	
Leucosporidium fellii	CBS 7287^T	AF189907	
Leucosporidium scottii	CBS 5930^T	AF070419	
Leuconostoc scottii	CBS 5932	AF189908	
Occultifur externus	IGC 4817^T	AF189909	
Occultifur externus	IGC 4557	AF189910	
Occultifur externus	IGC 4823	AF189911	
Reniforma strues	CBS 8263^T	AF189912	
Rhodosporidium babjevae	CBS 7808^T	AF070420	
Rhodosporidium babjevae	KB 649	AF189913	
Rhodosporidium diobovatum	CBS 6085^T	AF070421	
Rhodosporidium diobovatum	CBS 6084	AF189914	
Rhodosporidium fluviale	CBS 6568^T	AF070422	
Rhodosporidium kratochvilovae	CBS 7436^T	AF071436	
Rhodosporidium kratochvilovae	IGC 4818	AF189916	
Rhodosporidium kratochvilovae	IGC 4819	AF189917	
Rhodosporidium kratochvilovae	IGC 4793	AF189918	
Rhodosporidium lusitaniae	CBS 7604^T	AF070423	
Rhodosporidium paludigenum	CBS 6567	AF070424	
Rhodosporidium sphaerocarpum	CBS 5939^T	AF070425	
Rhodosporidium sphaerocarpum	CBS 5941	AF189919	
Rhodosporidium toruloides	CBS 349	AF070426	
Rhodotorula araucariae	CBS 6031^T	AF070427	
Rhodotorula armeniaca	CBS 8076^T	AF189920	
Rhodotorula aurantiaca	CBS 317^T	AF189921	
Rhodotorula auriculariae	CBS 6379^T	AF189922	
Rhodotorula bogoriensis	CBS 4101^T	AF189923	

Table 2 (*cont.*)

Strain	Strain no.*	GenBank accession no.	
		D1/D2	ITS†
Rhodotorula buffonii	CBS 2838^T	AF189924	
Rhodotorula creatinivora	CBS 8620^T	AF189925	
Rhodotorula cresolica	CBS 7998^T	AF189926	
Rhodotorula diffluens	CBS 5233^T	AF075485	
Rhodotorula ferulica	CBS 7402	AF189927	
Rhodotorula fujisanensis	CBS 4551^T	AF189928	
Rhodotorula fujisanensis	CBS 6371	AF189929	
Rhodotorula fujisanensis	CBS 8264	AF189930	
Rhodotorula futronensis‡	CBS 8163^T	AF189931	
Rhodotorula foliorum	CBS 6370	AF075499	
Rhodotorula fragraria	CBS 6254^T	AF070428	
Rhodotorula glutinis	CBS 20^T	AF070430	
Rhodotorula glutinis var. *dairenensis*	CBS 4406^T	AF070429	
Rhodotorula graminis	CBS 2826^T	AF070431	
Rhodotorula graminis	KB 650	AF189932	
Rhodotorula hordea	CBS 6403^T	AF189933	
Rhodotorula ingeniosa	CBS 4240^T	AF189934	
Rhodotorula javanica	CBS 5236^T	AF189935	
Rhodotorula lactosa	CBS 5826^T	AF189936	
Rhodotorula laryngis	CBS 2221^T	AF189937	AF190014
Rhodotorula laryngis	IGC 4886	AF189938	
Rhodotorula laryngis	Y-17494	AF189939	
Rhodotorula laryngis	Y-17503	AF189940	
Rhodotorula laryngis	Y-17504	AF189941	
Rhodotorula zsoltii‡	CBS 5695^T	AF189942	AF190013
Rhodotorula lignophila	CBS 7109^T	AF189943	
Rhodotorula marina	CBS 2365^T	AF189944	
Rhodotorula minuta	CBS 319^T	AF189945	AF190011
Rhodotorula minuta	CBS 4408	AF189946	
Rhodotorula minuta	CBS 7296	AF189947	
Rhodotorula texensis‡	CBS 2177^T	AF189948	AF190010
Rhodotorula tokyoensis‡	CBS 4407^T	AF189949	AF190012
Rhodotorula mucilaginosa	CBS 316^T	AF070432	
Rhodotorula mucilaginosa	IGC 4349	AF189951	
Rhodotorula mucilaginosa	Y-17485	AF189952	
Rhodotorula mucilaginosa	Y-17493	AF189953	
Rhodotorula mucilaginosa	Y-17495	AF189954	
Rhodotorula mucilaginosa	Y-17496	AF189955	
Rhodotorula mucilaginosa	Y-17499	AF189956	
Rhodotorula mucilaginosa	Y-17500	AF189957	
Rhodotorula mucilaginosa	Y-17501	AF189958	
Rhodotorula mucilaginosa	CBS 8383	AF189959	
Rhodotorula rubra‡	CBS 17^T	AF189960	
Sporobolomyces alborubescens‡	CBS 482^T	AF189961	
Rhodotorula muscorum	CBS 6921^T	AF070433	
Rhodotorula nothophagi	CBS 8166^T	AF189950	
Rhodotorula pallida	CBS 320^T	AF189962	
Rhodotorula philyla	CBS 6272^T	AF075471	
Rhodotorula pilati	CBS 7039^T	AF189963	

Table 2 (*cont.*)

Strain	Strain no.*	GenBank accession no.	
		D1/D2	ITS†
Rhodotorula pustula	CBS 6527^T	AF189964	
Rhodotorula slooffiae	CBS 5706^T	AF189965	
Rhodotorula slooffiae	CBS 7093	AF189966	
Rhodotorula slooffiae	CBS 7095	AF189967	
Rhodotorula slooffiae	IGC 4887	AF189968	
Rhodotorula sonckii	CBS 6713^T	AF189969	
Rhodotorula vanillica	CBS 7404^T	AF189970	
Rhodotorula yarrowii	CBS 7417^T	AF189971	
Sakaguchia dacryoidea	CBS 6353^T	AF189972	
Sakaguchia dacryoidea	CBS 6356	AF189973	
Sphacelotheca polygoni-persicariae	IGC 4293	AF189974	
Sporidiobolus microsporus	CBS 7041^T	AF070436	
Sporidiobolus johnsonii	CBS 5470^T	AF070435	
Sporidiobolus johnsonii	CBS 2630	AF189976	
Sporobolomyces holsaticus‡	CBS 1522^T	AF189975	
Sporidiobolus pararoseus	CBS 491^T	AF189977	
Sporobolomyces ruber‡	CBS 4216^T	AF189978	
Sporobolomyces pararoseus‡	CBS 484^T	AF070437	
Sporidiobolus ruineniae	CBS 5001^T	AF070438	
Sporidiobolus ruineniae var. *coprophilus*	CBS 5811^T	AF070434	
Sporidiobolus salmonicolor	CBS 490^T	AF070439	
Sporidiobolus salmonicolor	Y-17498	AF189979	
Sporobolomyces coprosmae	CBS 7899^T	AF189980	
Sporobolomyces coprosmicola	CBS 7897^T	AF189981	
Sporobolomyces dracophylli	CBS 7900^T	AF189982	
Sporobolomyces elongatus	CBS 8080^T	AF189983	
Sporobolomyces falcatus	CBS 7368^T	AF075490	
Sporobolomyces foliicola	CBS 8075^T	AF189984	
Sporobolomyces gracilis	CBS 71^T	AF189985	
Sporobolomyces griseoflavus	CBS 7284^T	AF189986	
Sporobolomyces inositophilus	CBS 7310^T	AF189987	
Sporobolomyces kluyveri-nielii	CBS 7168^T	AF189988	
Sporobolomyces lactophilus	CBS 7527^T	AF177411	
Sporobolomyces linderae	CBS 7893^T	AF189989	
Sporobolomyces marcillae	CBS 4217^T	AF070440	
Sporobolomyces oryzicola	CBS 7228^T	AF189990	
Sporobolomyces phyllomatis	CBS 7198^T	AF189991	
Sporobolomyces roseus	CBS 486^T	AF070441	
Sporobolomyces ruber	CBS 7512^T	AF189992	
Sporobolomyces ruberrimus	CBS 7500^A	AF070442	
Sporobolomyces ruberrimus var. *albus*‡	CBS 7501^A	AF189993	
Sporobolomyces ruberrimus var. *albus*	CBS 7253	AF189994	
Sporobolomyces salicinus	CBS 6983^T	AF189995	
Sporobolomyces sasicola	CBS 7285^T	AF177412	
Sporobolomyces singularis	CBS 5109^T	AF189996	
Sporobolomyces subbrunneus	CBS 7196^T	AF189997	
Sporobolomyces taupoensis	CBS 7898^T	AF177413	
Sporobolomyces tsugae	CBS 5038^T	AF189998	
Sporobolomyces xanthus	CBS 7513^T	AF177414	
Sterigmatomyces elviae	CBS 5922^T	AF177415	

Table 2 (*cont.*)

Strain	Strain no.*	GenBank accession no.	
		D1/D2	ITS†
Rhodotorula acuta‡	CBS 7053^T	AF189999	
Rhodotorula dulciaminis‡	CBS 7288^T	AF190000	
Sterigmatomyces halophilus	CBS 4609^T	AF177416	

* T, Type strain; A, authentic strain.

† Not all strains were examined in the ITS region.

‡ Species considered to be synonyms of the lead listed species as determined by sequence analysis and examination of classical taxonomic characteristics.

Table 3. Ustilaginomycetous yeasts examined in the D1/D2 rDNA regions

Strain	Strain no.*	Accession no.
Malassezia furfur	CBS 7019^T	AJ249955†
Malassezia globosa	CBS 7966^T	AJ249951†
Malassezia obtusa	CBS 7876^T	AJ249954†
Malassezia pachydermitis	CBS 1879^T	AJ249952†
Malassezia restricta	CBS 7877^T	AJ249950†
Malassezia slooffiae	CBS 7956^T	AJ249956†
Malassezia sympodialis	CBS 7222^T	AJ249953†
Rhodotorula acheniorum	CBS 6386^T	AF190001
Rhodotorula bacarum	CBS 6526^T	AF190002
Rhodotorula hinnulea	CBS 8079^T	AF190003
Rhodotorula phylloplana	CBS 8073^T	AF190004
Sympodiomycopsis paphiopedili	CBS 7429^T	AF190005

* T, Type strain.

† EMBL accession number.

are denoted by a 'T' following the strain number. The GenBank numbers for D1/D2 and ITS are listed in Tables 1–3. Not all strains were analysed in the ITS region. Sequences not included in the lists that are illustrated in Figs 1–3 were from the following sources: *Tremella* (Chen, 1998), smut and related fungi (Begerow *et al*., 1997), *Entyloma*, *Melanotaenium*, *Pseudozyma*, *Tilletiopsis*, *Tilletiaria* and *Ustilago* spp. (Boekhout *et al*., 1995).

Strains were obtained from the American Type Culture Collection (ATCC), ARS Culture Collection (NRRL), Peoria, IL (Y), Centraalbureau voor Schimmelcultures (CBS), the Portuguese Yeast Culture Collection (New University of Lisbon) (IGC), Brian Steffenson, North Dakota State University (KB) and Helen Vishniac, Oklahoma State University (*Cryptococcus consortionis*). Cells from pure cultures were grown for 12–24 h in GYP broth (2% glucose, 0·5% peptone and 0·1% yeast extract), then centrifuged/washed with distilled water and converted to sphaeroplasts by incubation for 2 h at 37 °C in 10 mM citrate buffer, pH 5·8, 1 M sorbitol and 10 mg ml^{-1} lysing enzymes from *Trichoderma harzianum* (Sigma), which was freshly prepared for each procedure. DNA was extracted and purified from the sphaeroplasts using a QIAamp tissue culture kit (Qiagen) according to the standard protocol. The DNA was amplified with the universal fungal primers ITS 5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and LR6 (5′-CGC CAG TTC TGC TTA CC-3′) using thermal cyclers (MJ Research). The resulting amplicon was purified with the QIAquick PCR purification kit (Qiagen).

Cycle sequencing of the D1/D2 600–650 bp region at the 5′ end of the LrDNA employed forward primer F63 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and reverse primer LR3 (5′-GGT CCG TGT TTC AAG ACG G-3′). ITS cycle sequencing primers included forward-strand primer ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and reverse-strand primer ITS4 (5′TCC TCC GCT TAT TGA TAT GC-3′). Nucleotide sequences were obtained using the standard Li-Cor protocol with IRD800 conjugate primers and a Li-Cor automated sequencer. All strains were examined with these techniques at the University of Miami; exceptions included strains of *Malassezia* that were sequenced at CBS using an ABI sequencer and protocol. Sequences were aligned with MEGALIGN (DNAstar) and visually corrected. Phylogenetic analysis employed PAUP* 4.0 using parsimony analysis, random step-wise addition and tree bisection–reconnection. Complete sequences are available in GenBank (Tables 1–3).

RESULTS AND DISCUSSION

Basidiomycetous yeasts are characterized by electron-dense and layered cell walls (Kreger-van Rij & Veenhuis, 1971; Simmons & Ahearn, 1987) and septal morphology, which has been used as a primary phylogenetic character (Boekhout *et al*., 1998; Moore, 1998). Hyphal states of species belonging to the Urediniomycetes have septa with 'simple' pores in which the cell wall is attenuated towards the central pore. Usually a single pore is observed, but multiple pores occur in *Kriegeria eriophori* (McLaughlin *et al*., 1995). Cell wall composition in the Urediniomycetes is dominated by mannose, glucose is present, fucose and rhamnose may be present and xylose is absent (Prillinger *et al*., 1991). Urediniomycetous yeasts are characterized by an absence of starch-like compounds and an inability to utilize inositol. Ustilaginomycetous taxa have 'micropore-like' septa, which may or may not have an inflated margin and which differ from 'simple' pores because they do not have tapering cell walls and probably lack a true pore (Bauer *et al*., 1989,

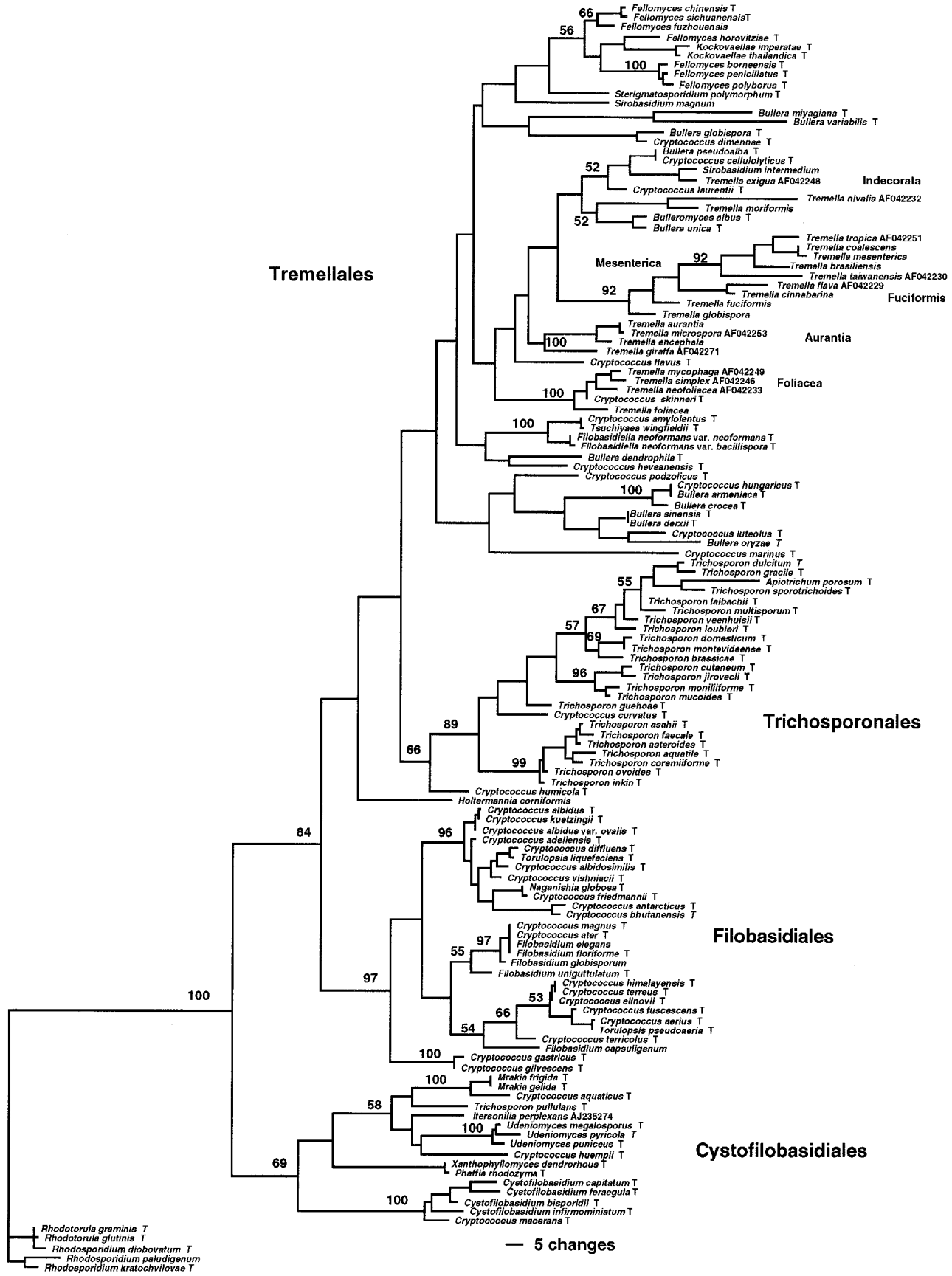

Fig. 1. For legend see facing page.

1997; Boekhout *et al*., 1992, 1998; O'Donnell & McLaughlin, 1984). The ustilaginomycetous yeast cell walls contain dominant levels of glucose, galactose and mannose are present and xylose is absent (Prillinger *et al*., 1990). Inositol may or may not be utilized; starch-like compounds are not produced. The hymenomycetous yeasts, in contrast, have dolipore septa and the cell walls contain glucose, mannose and xylose (Prillinger *et al*., 1991, 1993; Roeijmans *et al*., 1998; Weijman & Golubev, 1987). Inositol is usually assimilated and starch-like compounds are produced by a majority of the hymenomycetous yeasts. Our molecular studies confirm the phylogenetic principles developed with the small-subunit rDNA (Swan & Taylor, 1995a, b, c; Swann *et al*., 1999) that yeasts are distributed among three classes. Consequently, our data are presented in trees that represent yeasts associated with the Hymenomycetes (Fig. 1), the Urediniomycetes (Fig. 2) and the Ustilaginomycetes (Fig. 3).

Yeast species of the Tremellomycetidae of the Hymenomycetes

On the basis of sequence analysis of the small-subunit rDNA, Swann & Taylor (1995c) recommended two subclasses among the Hymenomycetes: (1) the Hymenomycetidae, containing the non-yeast-like macrofungi, including the mushrooms and puffballs; and (2) the Tremellomycetidae. As a result of our analysis of the D1/D2 region, the hymenomycetous yeasts are presented in four major clades of the Tremellomycetidae (Fig. 1): the Tremellales, the Trichosporonales, the Filobasidiales and the Cystofilobasidiales. The hymenomycetous yeast genus, *Cryptococcus*, is polyphyletic and occurs in all four clades. The remaining genera occur in single clades: (1) the Tremellales – *Bullera*, *Bulleromyces*, *Fellomyces*, *Filobasidiella*, *Kockovaella* and *Tsuchiyaea*; (2) the Trichosporonales – all species of *Trichosporon* with the exception of *Trichosporon pullulans*, which occurs in the Cystofilobasidiales; (3) the Filobasidiales – *Filobasidium*; and (4) the Cystofilobasidiales – *Cystofilobasidium*, *Mrakia*, *Phaffia*, *Udeniomyces* and *Xanthophyllomyces*.

The Tremellales clade

The Tremellales consists of seven families (Wells, 1994) but the molecular systematics of this order has not been established. Our study concentrated on the occurrence of yeasts in this order. In addition, we included species of the Tremellaceae (*Tremella* spp. and *Holtermannia corniformis*) and two species of the Sirobasidiaceae (*Sirobasidium magnum* and *Sirobasidium intermedium*). The Tremellales clade and many of the internal clusters do not have bootstrap support, which may reflect the heterogeneity of the order and/or the incomplete sampling of taxa. The major teleomorphic representative of the Tremellales included in this analysis is the genus *Tremella*. Our view of *Tremella* is preliminary, as the analysis covers only 20 of the estimated 120 species (Bandoni, 1995). Chen (1998) demonstrated five clusters among the species of *Tremella*, which are indicated in Fig. 1. *Cryptococcus skinneri* is in the Foliacea cluster, which has strong (100%) statistical support. The Indecorata cluster that lacks statistical support includes *Bulleromyces albus*, *Bullera unica*, *Bullera pseudoalba*, *Cryptococcus cellulolyticus* and *Cryptococcus laurentii*.

There are two distinct teleomorphic yeast genera in the Tremellales, namely *Bulleromyces* and *Filobasidiella*. A third genus, *Sterigmatosporidium*, has been described as a teleomorph; however, the apparent absence of a tremellaceous basidium suggests that a further investigation of the life cycle is required. The 2–4-celled basidial morphology of *Bulleromyces* is similar to that of many of the Tremellales (Boekhout *et al*., 1991). The anamorph of *Bulleromyces* is in the genus *Bullera* (Boekhout & Nakase, 1998), which only occurs in the Tremellales. Based on distributions of species within the Tremellales, many of the *Cryptococcus* species appear to be related to *Bullera* spp., for example *Bullera pseudoalba*/*C. cellulolyticus* and *Bullera armeniaca*/*Cryptococcus hungaricus*. A major taxonomic distinction between *Bullera* and *Cryptococcus* is the production of ballistoconidia by *Bullera*, which, based on these relationships, does not appear to be a phylogenetically significant character. A similar conclusion can be reached for the cluster of stalked-conidia-forming genera *Kockovaella* and *Fellomyces*, which differ by the presence or absence of ballistoconidia.

In contrast to previous concepts, which placed *Filobasidiella* among the Filobasidiales (Bandoni, 1995; Boekhout *et al*., 1998), the human pathogens *Filobasidiella neoformans* var. *neoformans* and var. *bacillispora* form a statistically supported (100%) cluster within the Tremellales. The sexual cycle of this species is distinct from the typical tremellaceous yeasts because of the presence of a slender, cylindrical, capitate holobasidium with basipetally formed chains of basidiospores (Kwon-Chung, 1998).

The Trichosporonales clade

The Trichosporonales clade (with bootstrap support of 89%) contains all of the species of *Trichosporon*, except for *Trichosporon pullulans*, which is located

Fig. 1. Hymenomycetous yeasts: phylogenetic analysis of the D1/D2 region of the large-subunit rDNA (one of 100 equally parsimonious trees). Number of characters, 651; constant characters, 285; parsimony-uninformative characters, 71; parsimony-informative characters, 295. Tree length, 2163; consistency index, 0·2779; homoplasy index, 0·7721. Numbers on branches, bootstrap percentages from 100 full heuristic bootstrap replications. The Tremellales cluster names are from Chen (1998). Species with GenBank numbers represent sequences obtained from GenBank; see Table 1 for the GenBank numbers of strains sequenced for this study. T, Type strain.

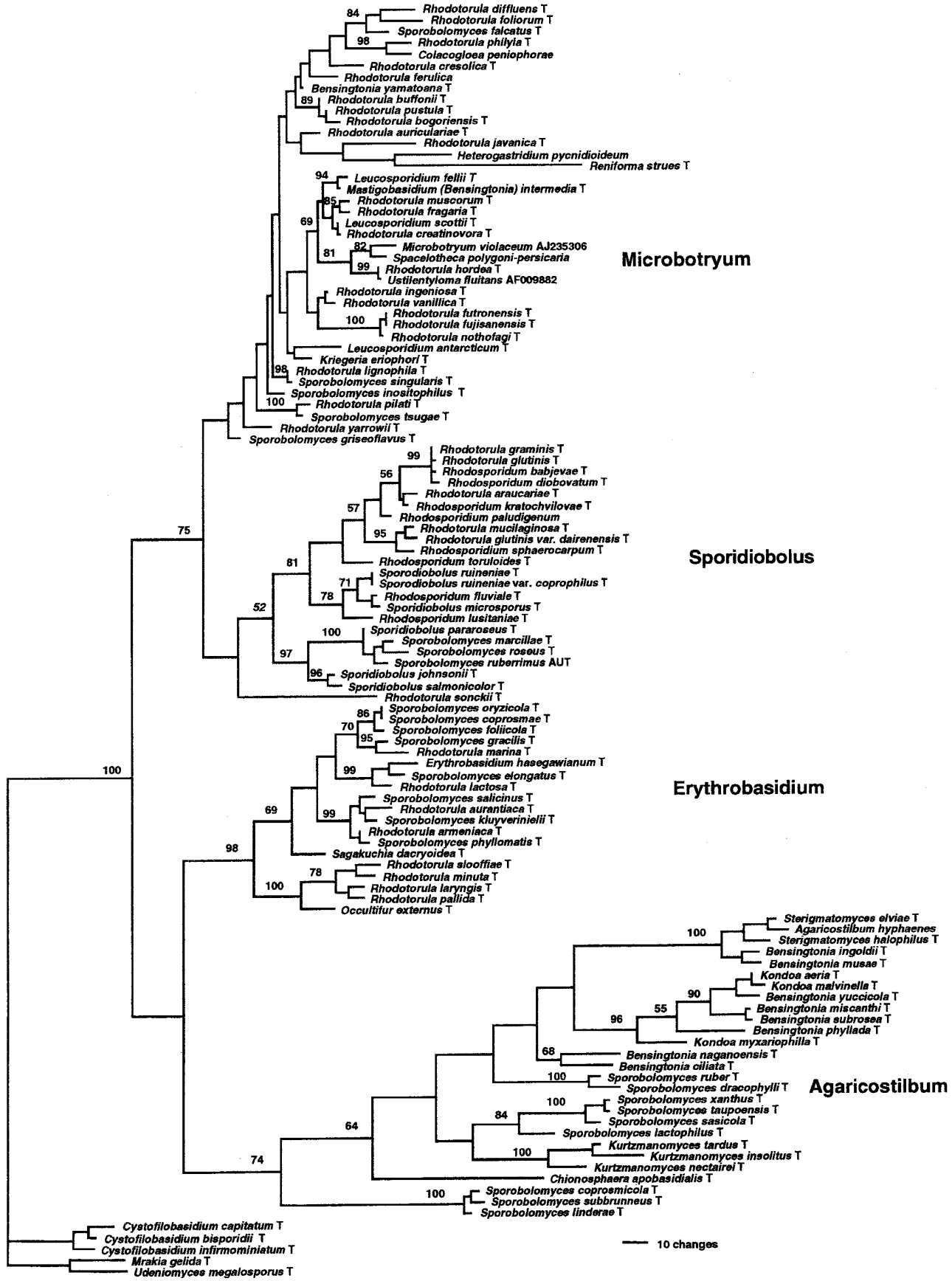

Fig. 2. For legend see facing page.

among the Cystofilobasidiales. The genus *Trichosporon*, which is characterized by the presence of arthroconidia, has been studied in detail by Guého *et al*. (1992, 1993) and includes the medically important species *Trichosporon asahii*, *Trichosporon cutaneum*, *Trichosporon inkin* and *Trichosporon mucoides*. Two additional species (*C. curvatus* and *Apiotrichum porosum*) that do not produce arthroconidia are found in this clade. The strain of *A. porosum* that we examined produced extensive hyphae and lacked a yeast-like phase. *Cryptococcus curvatus* produces pseudohyphae and yeast cells that range in shape from ovoid to elongate. A third species, *Cryptococcus humicola*, is attached to the clade (66% bootstrap support). This species, which has a distinct yeast phase, produces extensive pseudo and true mycelium; arthroconidia, however, have not been observed. Further investigations on these *Cryptococcus* spp. and *A. porosum* are required to develop an understanding of their phylogenetic relationships to the genus *Trichosporon*.

The morphological and molecular characteristics of the genus *Trichosporon* demonstrate that the clade is phylogenetically distinct from the hymenomycetous sister clades Tremellales, Filobasidiales and Cystofilobasidiales. Consequently, the new order Trichosporonales is proposed.

Trichosporonales Boekhout *et* Fell ord. nov. Fungi hymenomycetales vel levadiniformes, anamorphici. Hyphae verae plerumque modo arthroconidiorum fragmentatae. Septa plerumque doliporis perforata; parenthesomatibus vulgo structuris tubularibus vel vesicularibus instructa. Parietes xylosum continentes. Coenzyma Q_9 vel Q_{10}.

Typus: *Trichosporon* Behrend. Anamorphic, hymenomycetous yeasts or yeast-like fungi. True hyphae proliferating by arthroconidia. Septa with dolipores, which may or may not have tubular/vesicular parenthesomes; cell walls with xylose; coenzyme Q_9 or Q_{10}. Type: *Trichosporon* Behrend. This order, which forms a coherent clade (with 89% bootstrap support), is a sister group of the Tremellales. Species included are: *Trichosporon asahii*, *Trichosporon asteroides*, *Trichosporon aquatile*, *Trichosporon brassicae*, *Trichosporon coremiiforme*, *Trichosporon cutaneum*, *Trichosporon domesticum*, *Trichosporon dulcitum*, *Trichosporon faecale*, *Trichosporon gracile*, *Trichosporon guehoiae*, *Trichosporon inkin*, *Trichosporon jirovecii*, *Trichosporon laibachii*, *Trichosporon loubieri*, *Trichosporon moniliiforme*, *Trichosporon montevideense*, *Trichosporon mucoides*, *Trichosporon multisporum*, *Trichosporon ovoides*, *Trichosporon veenhuisii*, *Apiotrichum porosum*, *Cryptococcus curvatus* and possibly *Cryptococcus humicola*. rDNA sequences indicate that *Trichosporon pullulans* belongs to the Cystofilobasidiales (Fell *et al*., 1999).

The Filobasidiales clade

The order Filobasidiales originally included the genera *Cystofilobasidium*, *Filobasidiella*, *Filobasidium*, *Mrakia* and *Xanthophyllomyces* (Bandoni, 1995; Boekhout *et al*., 1998; Wells, 1994). Swann & Taylor (1995c) recommended a reassessment of the Filobasidiales, indicating that *Filobasidiella* was more closely related to *Tremella* than to *Filobasidium*; in addition, *Cystofilobasidium* and *Mrakia* did not form a monophyletic group with *Filobasidium*. The data presented in Fig. 1 concur with the Swann & Taylor conclusions. The Filobasidiales clade has bootstrap support of 97%. The only teleomorphic genus in this clade is *Filobasidium*, whose sexual cycle differs from that of *Filobasidiella* by the formation of petal-like whorls of basidiospores at the apex of a slender holobasidium (Kwon-Chung, 1998). The Filobasidiales clade is composed of four clusters, though they do not all have strong bootstrap support. One cluster includes *Cryptococcus albidus* and related species, as well as several Antarctic species (*Cryptococcus albidosimilis*, *Cryptococcus antarcticus*, *Cryptococcus friedmannii* and *Cryptococcus vishniacii*). The second cluster consists of *Cryptococcus ater* and the members of *Filobasidium*, with the exception of *Filobasidium capsuligenum* (which is found in the third cluster with *Cryptococcus aerius*, *Cryptococcus terreus* and related species). The fourth cluster consists of *Cryptococcus gastricus* and *Cryptococcus gilvescens*. The necessary emendation of the order Filobasidiales is premature, as members of the family Syzygosporaceae were not analysed.

The Cystofilobasidiales clade

The recently described order Cystofilobasidiales (Fell *et al*., 1999) has teliospores, which is a unique feature among the teleomorphic Hymenomycetes. Generally, this type of probasidium is found among the urediniomycetous yeasts. Other major characteristics of the Cystofilobasidiales include holobasidia and hyphal septa with dolipores that lack parenthesomes. The teliospore-forming genera *Mrakia* and *Cystofilobasidium* are located in two distinct clusters. Anamorphic genera include the ballistoconidia-forming *Udeniomyces*, the arthroconidia-forming *T. pullulans* and several species of *Cryptococcus*. *Xantho-*

Fig. 2. Urediniomycetous yeasts, representing four clades (*Microbotryum*, *Sporidiobolus*, *Erythrobasidium* and *Agaricostilbum*): phylogenetic analysis of the D1/D2 region of the large-subunit rDNA (one of 100 equally parsimonious trees). Number of characters, 657; constant characters, 241; parsimony-uninformative characters, 56; parsimony-informative characters, 360. Tree length, 2402; consistency index, 0·3118; homoplasy index, 0·6882. Numbers on branches, bootstrap percentages from 100 full heuristic bootstrap replications. Species with GenBank numbers represent sequences obtained from GenBank; see Table 2 for the GenBank numbers of strains sequenced for this study. T, Type strain.

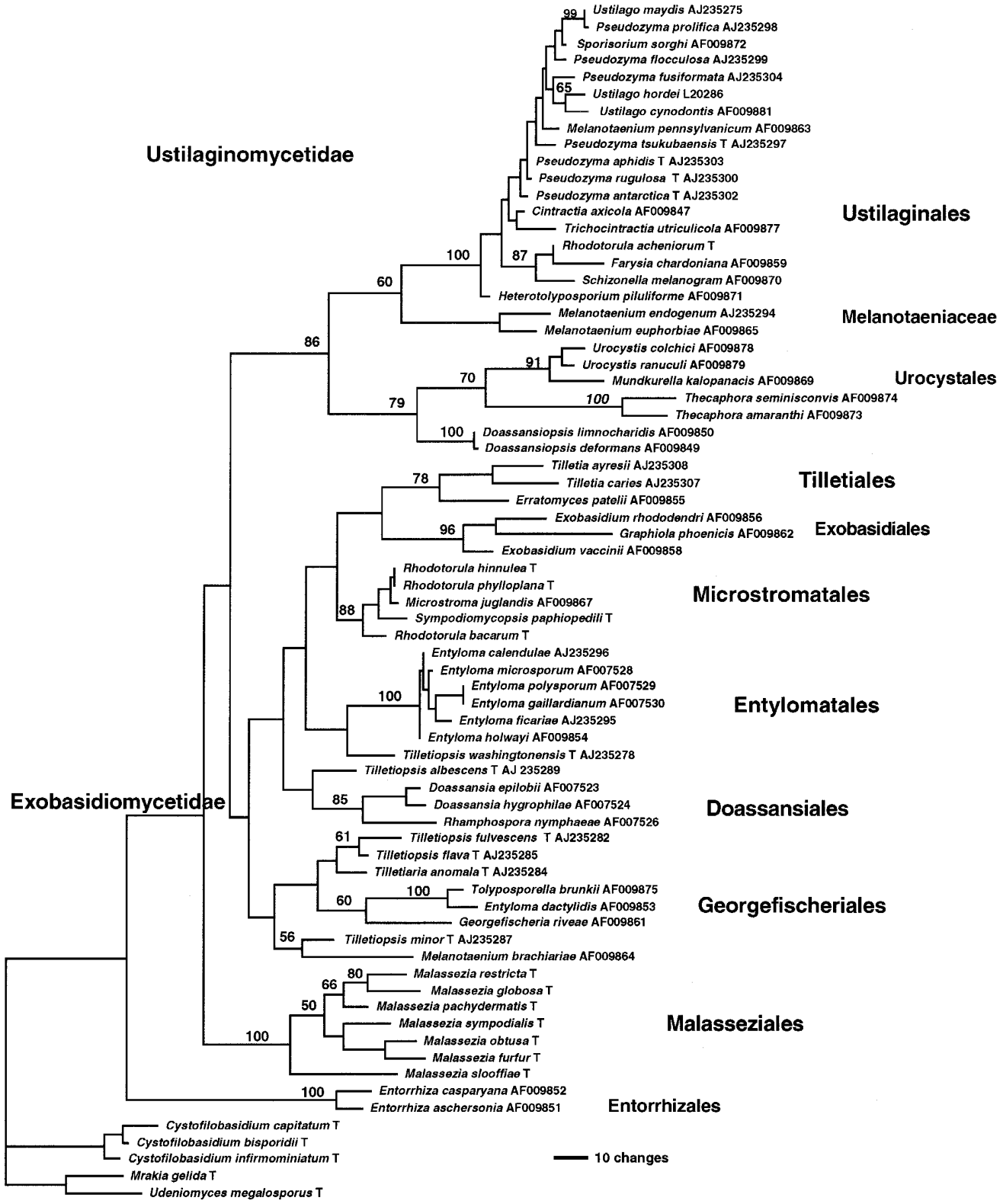

Fig. 3. Ustilaginomycetous fungi and associated yeasts: phylogenetic analysis of the D1/D2 region of the large-subunit rDNA (one of 100 equally parsimonious trees). Number of characters, 540; constant characters, 218; parsimony-uninformative characters, 37; parsimony-informative characters, 285. Tree length, 1555; consistency index, 0·3588; homoplasy index, 0·6412. Numbers on branches, bootstrap percentages from 100 full heuristic bootstrap replications. Names of orders are from Begerow *et al*. (1997). Species with GenBank numbers represent sequences obtained from GenBank; see Table 3 for the GenBank numbers of strains sequenced for this study. T, Type strain.

phyllomyces dendrorhous and *Phaffia rhodozyma* are important agro-industrial sources of astaxanthin. On the basis of sequence analysis of the intergenic spacer (IGS) and ITS regions, these two taxa have been reported to represent separate species (Fell & Blatt, 1999). The teleomorph (*Xanthophyllomyces*) produces holobasidia that do not arise from teliospores.

Yeast species of the Urediniomycetes

There are four major clades in this tree (Fig. 2), which are labelled for this discussion as the clades *Microbotryum*, *Sporidiobolus*, *Agaricostilbum* and *Erythrobasidium*. Three genera are in two or more clades. *Bensingtonia* occurs in the *Microbotryum* and *Agaricostilbum* clades; *Rhodotorula* is in the *Microbotryum*, *Sporidiobolus* and *Erythrobasidium* clades, but not the *Agaricostilbum* clade; *Sporobolomyces* is in all four clades. Genera that occur in single clades areas follows: (1) *Microbotryum* clade – *Leucosporidium*; (2) *Sporidiobolus* clade – *Rhodosporidium* and *Sporidiobolus*; (3) *Agaricostilbum* clade – *Kondoa*, *Kurtzmanomyces* and *Sterigmatomyces*; (4) *Erythrobasidium* clade – *Erythrobasidium*, *Sakaguchia* and *Occultifur*.

The *Microbotryum* clade

The *Microbotryum* and *Sporidiobolus* clades represent the Sporidiobolaceae of Boekhout *et al*. (1998) and the Microbotryomycetidae of Swann *et al*. (1999). The order Microbotryales was formally described by Bauer *et al*. (1997) as 'phytoparasitic members of the Basidiomycota having transversely septate basidia with multiple production of sessile basidiospores and only intercellular hyphae.' These authors divided the order into two families, i.e. the Microbotryaceae (with poreless septae) and the Ustilentylomataceae (with simple septal pores). The two families occur in a single cluster, represented by *Microbotryum violaceum* and *Ustilentyloma fluitans*, with 81% bootstrap support (Fig. 2). In addition to members of the Microbotryales, this clade includes species from two other orders: *Colacogloea peniophorae* and *Kriegeria eriophori* in the Platygoleales; and *Heterogastridium pycnidioideum* in the Heterogastridiales (Bandoni, 1995). Consequently, the *Microbotryum* clade is phylogenetically diverse, as reflected by the lack of bootstrap support for the clade and for many of the internal clusters. A unifying characteristic within this clade is the presence of colacosomes or lenticular bodies, which are an indication of mycoparasitism (Bauer & Oberwinkler, 1991; Bauer *et al*., 1997; Boekhout *et al*., 1992).

Yeast species from six genera are included in the *Microbotryum* clade: the teliospore-forming genera *Leucosporidium* and *Mastigobasidium* and species of the anamorphic genera *Rhodotorula*, *Sporobolomyces*, *Bensingtonia* and *Reniforma*. Golubev (1999) described *Mastigobasidium*, which is the sexual state of *Bensingtonia intermedia*. The latter species is the sole member of *Bensingtonia* that resides in the *Microbotryum* clade. *Mastigobasidium intermedium* is closely related to *Leucosporidium fellii* and the two species produce phragmometabasidia with bacilliform basidiospores that form in clusters on pegs (Statzell-Tallman & Fell, 1998; Golubev, 1999). This cluster characteristic is unique among the teliosporic yeasts, which usually produce single basidiospores or pairs of basidiospores per sporulation site. The formation of phragmometabasidia by *Leucosporidium* and *Mastigobasidium* is a characteristic shared with the teliospore-forming plant parasites *Microbotryum*, *Sphacelotheca* and *Kriegeria*.

The majority of the yeasts in the *Microbotryum* clade produce white- to cream-coloured colonies, in contrast to the visible red pigments produced by species among the *Sporidiobolus* clade. An exception is *Rhodotorula fujisanensis*, whose colonies may have a light pink colour (Johnson & Phaff, 1978; Sampaio & Fonseca, 1995). *Reniforma strues* is unique among the basidiomycetous yeasts because of the presence of kidney-shaped vegetative cells and coenzyme Q_7 (H. J. Roeijmans, personal communication); other yeasts in the Microbotryomycetidae contain coenzyme Q_9 or Q_{10}. The specific placement of *Reniforma strues* within the clade is questionable, as the apparent proximity to *H. pycnidioideum* may be a result of long-branch attraction in the parsimony analysis rather than a phylogenetic relationship.

The *Sporidiobolus* clade

The *Sporidiobolus* clade represents the red-pigmented teliosporic yeasts *Rhodosporidium* and *Sporidiobolus* with phragmometabasidia, and their related anamorphs in the genera *Rhodotorula* and *Sporobolomyces*. Although pigment chemistry is usually considered to be an unreliable characteristic in systematics, the presence of carotenoid pigments appears to differentiate the *Sporidiobolus* and *Microbotryales* clades. Species in the *Sporidiobolus* clade do not produce extracellular starch-like compounds or utilize D-glucuronate; coenzyme Q_{10} is usually present. In this clade, there are two major clusters with significant statistical support: the *Rhodotorula graminis* cluster (81% support) and the *Sporidiobolus pararoseus* cluster (98% support). The *Rhodotorula graminis* cluster consists of two branches: (1) the non-ballistoconidial species of *Rhodotorula* and *Rhodosporidium*; and (2) the *Sporidiobolus ruineniae* branch that includes the ballistoconidia-positive (*Sporidiobolus*) and -negative (*Rhodosporidium*) species. The unique characteristic of species in this branch, in contrast to other members of the *Sporidiobolus* clade, is the formation of phragmometabasidia on pronounced stalks. Species in the *Sporidiobolus pararoseus* cluster produce ballistoconidia and develop phragmometabasidia that lack stalked connections to the teliospore.

The species composition of the *Sporidiobolus* clade (Fig. 2) conforms with the core species of the Sporidiales as presented by Swann & Taylor (1995b),

with the exception of *Leucosporidium scottii* and *H. pycnidioideum*, which are recognized (Fig. 2) as members of the *Microbotryum* clade. Moore (1980) distinguished two families in his concept of the Sporidiales: the Sporidiaceae and the Sporidiobolaceae, which are characterized by presence or absence of ballistoconidia. We do not accept these families, because of the close relationship between ballistoconidia-forming and non-ballistoconidia-forming species (as exemplified by the branch arrangements of *Rhodosporidium fluviale*/*Sporidiobolus microsporus* and *Rhodosporidium lusitaniae*/*Sporidiobolus ruineniae*). The biological uniformity of the *Sporidiobolus* clade indicates that this group should be formally recognized as an order. However, the weak (52%) statistical support suggests the need for further investigation with additional taxa.

The *Erythrobasidium* clade

The *Erythrobasidium* clade, as coined by Swann & Taylor (1955a), is strongly supported (98%) with four significant internal clusters. This clade includes pigmented species of *Rhodotorula*, *Sporobolomyces*, *Sakaguchia*, *Erythrobasidium* and *Occultifur*. Sexual cycles within the clade differ. *Sakaguchia* (*Rhodosporidium*) *dacryoideum* produces teliospores that germinate to a 2–4-celled metabasidium with repetitively budding basidiospores. *Erythrobasidium* produces holobasidia directly from dikaryotic hyphae. *Occultifur externus* (Sampaio *et al*., 1999) is a non-teliospore-forming yeast that produces auricularioid basidia with ballistospores. The strong statistical support indicates that further biological study will develop criteria for formal classification of the clade.

The *Agaricostilbum* clade

The *Agaricostilbum* clade is comprised of several strongly supported clusters, which demonstrate some generic and phenotypic separation. The clade is largely composed of ballistoconidia-forming species of the genera *Bensingtonia* and *Sporobolomyces*. The several branches that include *Bensingtonia*, *Sterigmatomyces* and *Kondoa* have the unified characteristic of coenzyme Q_9, in contrast to the *Sporobolomyces*, *Kurtzmanomyces* branches that exhibit the presence of coenzyme Q_{10}.

There is morphological variability within the *Agaricostilbum* clade. Species in the genus *Agaricostilbum* inhabit palms and produce synnemata-like basidiomata. *Chionosphaera apobasidialis*, which occurs on a long branch in the *Agaricostilbum* clade, produces a basidiocarp that is terminally located on synnemata composed of dikaryotic hyphae. *Kondoa* was originally described with teliospores, but an evaluation of the life cycle of *Kondoa* revealed the formation of auricularoid basidia with ballistospores in the absence of teliospore production (Fonseca *et al*., 2000a). *Sterigmatomyces* is an anamorphic genus in the same cluster with *Agaricostilbum hyphaenes* (bootstrap support of 100%). The yeast cells of *Sterigmatomyces* form distinct stalks with terminal conidia and a mid-stalk conidial separation. *Kurtzmanomyces* is similar to *Sterigmatomyces* in terms of the formation of conidia on stalks; however, conidial separation in *Kurtzmanomyces* is at the distal end of the stalk. In addition, some of the *Sporobolomyces* species in this cluster (particularly *Sporobolomyces lactophilus*) can reproduce sympodially with conidia on a long stalk.

Distribution of yeasts among the Ustilaginomycetes

Fig. 3 consists of the D1/D2 sequences from yeast species in our study combined with data on the plant associated fungi in the Ustilaginomycetes by Begerow *et al*. (1997), *Pseudozyma*, *Tilletia* and *Tilletiopsis* spp. by Boekhout *et al*. (1995). Begerow *et al*. (1997) separated the Ustilaginomycetes into the clades, as depicted in Fig. 3. The majority of the yeast species examined, which were originally isolated from plants, are distributed into several clades: *Pseudozyma* spp. and *Rhodotorula acheniorum* are in the Ustilaginales of the Ustilaginomycetidae. *Rhodotorula bacarum*, *Rhodotorula hinnulea*, *Rhodotorula phylloplana* and *Sympodiomycopsis paphiopedili* are in the Microstromatales of the Exobasidiomycetidae. *Tilletia* is phylogenetically associated with the Tilletiales. *Tilletiopsis* is found among the Entylomatales, Doassaniales and Georgefischeriales.

Species of *Malassezia* appear in a statistically supported (100%) clade that is not included in either the Ustilaginomycetidae or the Exobasidiomycetidae. The genus is distinct among the Ustilaginomycetes due to a general association with humans and other animals and because of a growth requirement, in many of the strains, for fatty acids. Begerow *et al*. (1999) proposed the separate order Malasseziales. For details of the genus, see Guého *et al*. (1996) and Guillot & Guého (1995).

Discrimination of phenotypically similar species by sequence analysis

Strain variation in characteristics, such as carbon- and nitrogen-utilization patterns, is a critical problem associated with identifications based on classical phenotypic characteristics. This variability has resulted in long lists of synonyms for some taxa, particularly the anamorphic species. Studies have been designed to examine the validity of these synonyms. For example, taxonomic relationships among three varieties of *C. albidus* (var. *albidus* var. *diffluens* and var. *aerius*) were examined by comparisons of the composition of capsular polysaccharides, serological differences, G+C content, DNA relatedness and whole-cell protein electrophoretic patterns (Ikeda *et al*., 1982; Shinoda *et al*., 1980; Sugita *et al*., 1992; Vancanneyt *et al*., 1994; Vaughan-Martini, 1991). The resulting consensus was that the type strains of these varieties represented distinct taxa. We examined this concept by D1/D2-sequence analysis of the type

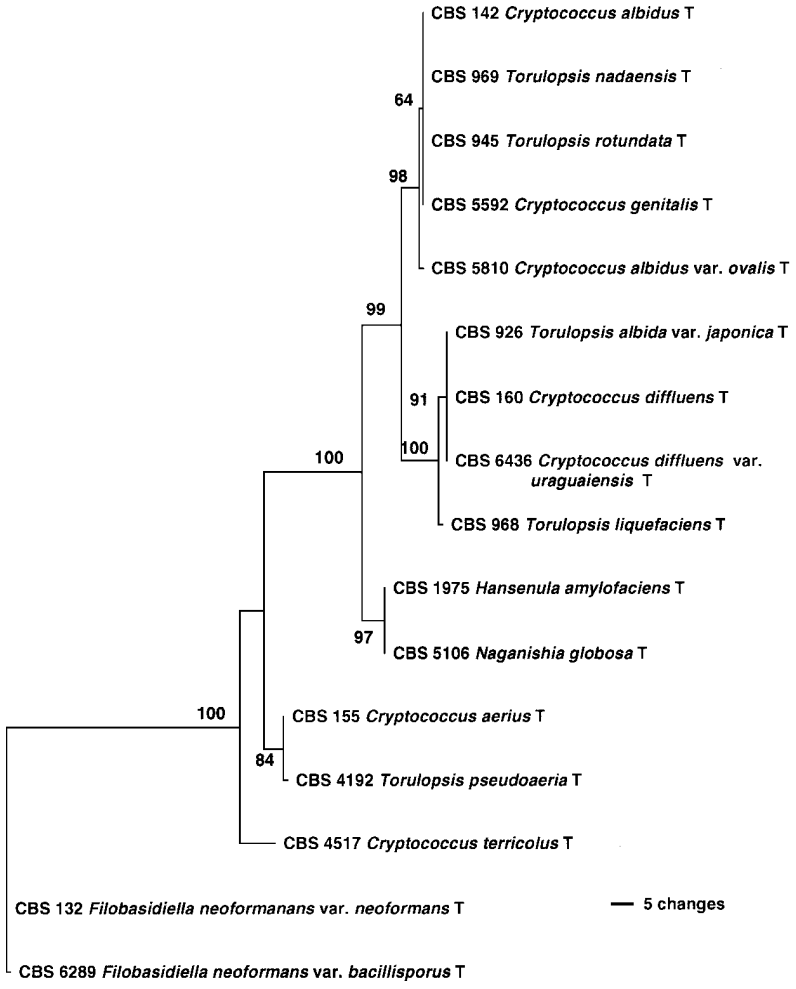

Fig. 4. *Cryptococcus albidus* and species listed as synonyms (Barnett *et al.*, 1990; Fell & Statzell-Tallman, 1998): phylogenetic analysis of the D1/D2 region of the large-subunit rDNA (one of three equally parsimonious trees). Number of characters, 631; constant characters, 519; parsimony-uninformative characters, 11; parsimony-informative characters, 101. Tree length, 143; consistency index, 0·8671; homoplasy index, 0·1329. Numbers on branches, bootstrap percentages from 1000 branch and bound replications. T, Type strain.

strains for 13 species listed as synonyms of *C. albidus* (Barnett *et al.*, 1990; Fell & Statzell-Tallman, 1998). The results (Fig. 4) indicated the presence of six distinct taxa. (1) *C. albidus* with three synonyms (*T. nadaensis*, *T. rotundata* and *C. genitalis*) and a phenotypically similar variety, i.e. *C. albidus* var. *ovalis*, which differs from *C. albidus* at one base position in the D1/D2 region and three base positions in the ITS region. These differences suggest genotypically distinct taxa or strains as observed within the genus *Mrakia* (Diaz & Fell, 2000) and between strains of *Xanthophyllomyces* (Fell & Blatt, 1999). (2) *Cryptococcus diffluens* with the synonyms *Cryptococcus diffluens* var. *uruguaiensis* and *Torulopsis gelatinosa*. (3) *Torulopsis liquefaciens*. (4) *Hansenula amylofaciens* and *Naganishia globosa*, which were originally considered to be teleomorphic ascomycetes (Dietrichson, 1954; Goto, 1963). The sequence alignment indicates that they represent an anamorphic basidiomycetous species that should be validated. (5) *Cryptococcus aerius* and the synonym *Torulopsis pseudoaeria*. (6) *Cryptococcus terricolus*. The taxonomic status of these species and other strains related to *Cryptococcus albidus* is addressed in a separate communication (Fonseca *et al.*, 2000b).

In similar studies, Hamamoto *et al.* (1987) examined *Rhodotorula minuta* and several synonyms with DNA hybridizations: they reported high levels of relative binding of *R. minuta* with *Rhodotorula texensis* (65%) and *Rhodotorula tokyoensis* (98%) and low levels with *Rhodotorula zsoltii* (31%), *Rhodotorula pallida* (24%) and *Rhodotorula marina* (18%). We examined *R. minuta* and seven synonyms (Fig. 5) and found that this complex represented five distinct species (*R. minuta*, *Rhodotorula slooffiae*, *R. pallida*, *Rhodotorula laryngis* and *R. marina*). *R. minuta* has two synonyms, namely *R. texensis* and *R. tokyoensis*. *R. minuta* differs by one nucleotide in the D1/D2 region from *R. texensis* and *R. tokyoensis* but the ITS sequences are identical, suggesting that the three strains represent a single taxon. Similarly, *R. zsoltii* is a synonym of *R. laryngis*, as the LrDNA and ITS sequences of the two taxa are identical.

ITS regions for species separations

As discussed for the *C. albidus* and *R. minuta* examples, strains with identical D1/D2 sequences are considered to represent a single species. The D1/D2 data generally

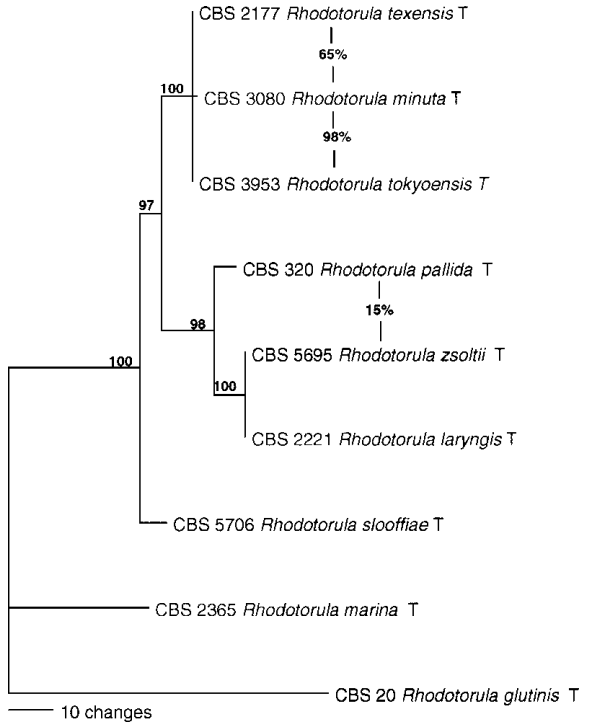

Fig. 5. *Rhodotorula minuta* and species listed as synonyms (Barnett *et al.*, 1990; Fell & Statzell-Tallman, 1998): phylogenetic analysis of the D1/D2 region of the large-subunit rDNA (single most parsimonious tree). Number of characters, 630; constant characters, 487; parsimony-uninformative characters, 89; parsimony-informative characters, 54. Tree length, 178; consistency index, 0·9101; homoplasy index, 0·0899. Numbers on branches, bootstrap percentages from 1000 branch and bound replications. Percentages between species, DNA relatedness (%) from Hamamoto *et al.* (1987). T, Type strain.

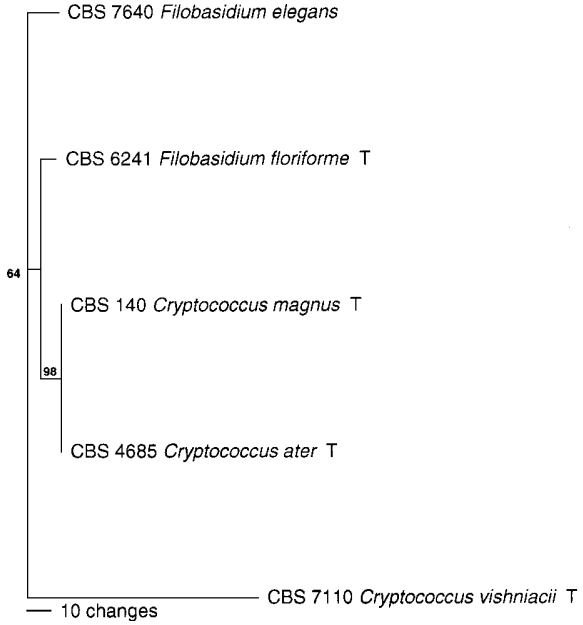

Fig. 6. *Filobasidium elegans* and related species as viewed by sequence analysis of the internal transcribed spacer region (single most parsimonious tree). Number of characters, 658; constant characters, 531; parsimony-uninformative characters, 113; parsimony-informative characters, 14. Tree length, 138; consistency index, 0·9855; homoplasy index, 0·0145. Numbers on branches, bootstrap percentages from 1000 branch and bound replications. T, Type strain. Bar, 10 changes.

agree with available DNA hybridization results and standard phenotypic data. There are situations, however, in which mating genetics and standard phenotypic characteristics indicate that strains with identical D1/D2 sequences represent separate species. *F. neoformans* varieties *neoformans* and *bacillispora* (the Tremellales clade) are a case in point. Differences between the two varieties, including physiology, geographical distributions, virulence, mating incompatibility systems and molecular genetics, have been extensively studied (Boekhout *et al.*, 1997, 1998; Fan *et al.*, 1994; Kwon-Chung, 1998). Our results, which concur with those studies, indicate the presence of two genetic entities; there is one base-position difference in the D1/D2 region and four differences in the ITS1 region but no differences in the 5.8 or ITS2 regions.

To further explore the use of the ITS region for species separations, we examined *C. ater*, *Cryptococcus magnus*, *Filobasidium elegans* and *Filobasidium floriforme*, whose D1/D2 sequences are identical (Fig. 1). *C. ater* and *C. magnus* are physiologically similar, although the two strains can be separated by their abilities to utilize D-glucosamine (Fell & Statzell-Tallman, 1998). A major difference between *C. ater*, *C. magnus* and *F. floriforme* is growth on nitrate and nitrite (Kurtzman & Fell, 1998). *F. elegans* is dissimilar from those three taxa in that it is unable to assimilate several compounds such as cellobiose, lactose, raffinose, melezitose, rhamnose, salicin and inositol (Fell & Statzell-Tallman, 1998; Kwon-Chung, 1998). The similarity of LrDNA sequences led Guého *et al.* (1993) to postulate that *C. ater* is an anamorphic state of *F. elegans*. ITS analysis (Fig. 6) presents a different picture: *C. ater* and *C. magnus* represent a single species (*C. magnus* has nomenclatural priority); *F. elegans*, *F. floriforme* and *C. magnus*, however, show significant differences in the ITS region, which confirms the presence of separate species. As indicated in Figs 1–3, there are several additional species with identical D1/D2 sequences that require ITS analysis (for example, *Sporobolomyces oryzicola*/*Sporobolomyces coprosmae*, *Rhodotorula futronensis*/*Rhodotorula fujisanensis* and *B. pseudoalba*/*C. cellulolyticus*).

Our experience, to date, suggests that strains that differed by two or more nucleotides in the D1/D2 region represented different taxa. In cases where we examined multiple strains within a species (Tables 1–3), D1/D2 sequences were identical. Taxonomic differences were not as clear when phenotypic analyses suggested distinct taxa, in contrast to the D1/D2 data, which were identical or differed by one nucleotide. The

examples we have presented indicate that the taxonomy can be clarified by ITS analysis. In addition to the ITS region, the IGS region is useful and may be required for strain separations as demonstrated with *Xanthophyllomyces*, *Phaffia* (Fell & Blatt, 1999) and *Mrakia* (Diaz & Fell, 2000).

Conclusions

A goal of the research was to examine the phylogenetic diversity of yeasts among the Ustilaginomycetes, Urediniomycetes and Hymenomycetes. The results confirm some accepted concepts; viz., the yeasts are a heterogeneous group of organisms and that many genera are artificial assemblages. For example, the genus *Cryptococcus* occurs in the following Hymenomycetes clades: Tremellales, Trichosporonales, Filobasidiales and Cystofilobasidiales. Similarly, species of *Rhodotorula* occur in the *Microbotryum*, *Sporidiobolus* and *Erythrobasidium* clades of the Urediniomycetes and the Ustilaginales and Microstromatales clades of the Ustilaginomycetes. In contrast, some genera such as *Kondoa*, *Cystofilobasidium* and *Fellomyces* may be phylogenetically distinct, as they are limited in distribution to specific clades and clusters. Consequently, the systematics of species must be interpreted in the context of the relationship to other species as viewed in these clusters and clades. A strength, therefore, of sequence analysis is that it provides testable hypotheses regarding the biology of these yeasts. One might anticipate that yeasts in clusters with *Kondoa*, *Sporidiobolus* or *Cystofilobasidium* will be found to exhibit similar phenotypic characteristics, such as cellular ultrastructure and life cycles. Similarly, study of the ustilaginaceous yeasts may reveal their biological relationships to the plant-parasitic and saprophytic filamentous fungi. In particular, are these yeasts anamorphic stages of parasitic filamentous teleomorphs or do they represent independent saprophytic members of this ecological niche?

Another goal of the research was to provide a method for determining yeast biodiversity. An understanding of the role and diversity of basidiomycetous yeasts in natural habitats has been slow to develop because of the difficulties of identification procedures. The urgent need for biodiversity studies is due to the worldwide rapid degradation of ecosystems. Industry and academics require precise identifications for various process- and physiologically/biochemically-oriented studies. Most species of yeasts can be directly identified by D1/D2 sequence analysis, alignment of the GenBank data, and placement within the appropriate phylogenetic tree. Alternatively, species can be identified via the ITS region, although a complete ITS database has not been developed and evaluated. Use of the database would have particular value for the phylogenetic placement of new, undescribed yeasts. In addition, through comparative analysis of the database, PCR primers and hybridization probes could be designated for the rapid identification of known species.

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