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*, *Rhodosporeidium*, *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 accom-

plish, 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†
<i>Apiotrichum porosum</i>	CBS 2040 ^T	AF189833	
<i>Bullera armeniaca</i>	CBS 7091 ^T	AF189883	
<i>Bullera crocea</i>	CBS 6714 ^T	AF075508	
<i>Bullera dendrophila</i>	CBS 6074 ^T	AF189870	
<i>Bullera derxii</i>	CBS 7225 ^T	AF189857	
<i>Bullera globispora</i>	CBS 6981 ^T	AF075509	
<i>Bullera miyagiana</i>	CBS 7526 ^T	AF189858	
<i>Bullera oryzae</i>	CBS 7194 ^T	AF075511	
<i>Bullera pseudoalba</i>	CBS 7227 ^T	AF075504	
<i>Bullera sinensis</i>	CBS 7238 ^T	AF189884	
<i>Bullera unica</i>	CBS 8290 ^T	AF075524	
<i>Bullera variabilis</i>	CBS 7347 ^T	AF189855	
<i>Bulleromyces albus</i>	CBS 501 ^T	AF075500	
<i>Cryptococcus adeliensis</i>	CBS 8351 ^T	AF137603	AF145328
<i>Cryptococcus aerius</i>	CBS 155 ^T	AF075486	AF145324
<i>Cryptococcus albidosimilis</i>	CBS 7711 ^T	AF137601	AF145325
<i>Cryptococcus albidosimilis</i>	ATCC 34633	AF137606	AF145331
<i>Cryptococcus albidus</i>	CBS 142 ^T	AF075474	AF145321
<i>Cryptococcus albidus</i>	IGC 2426	AF181514	
<i>Cryptococcus albidus</i>	IGC 4789	AF181531	
<i>Cryptococcus albidus</i>	IGC 4963	AF181509	
<i>Cryptococcus albidus</i>	IGC 4990	AF181511	
<i>Cryptococcus genitalis</i> ‡	CBS 5592 ^T	AF181538	
<i>Torulopsis nadaensis</i> ‡	CBS 969 ^T	AF181516	
<i>Torulopsis rotundata</i> ‡	CBS 945 ^T	AF181517	
<i>Cryptococcus albidus</i> var. <i>ovalis</i>	CBS 5810 ^T	AF137605	AF145329
<i>Cryptococcus amyloleptus</i>	CBS 6039 ^T	AF105391	
<i>Cryptococcus antarcticus</i>	CBS 7687 ^T	AF075488	AF145326
<i>Cryptococcus aquaticus</i>	CBS 5443 ^T	AF075470	
<i>Cryptococcus bhutanensis</i>	CBS 6294 ^T	AF137599	AF145317
<i>Cryptococcus cellulolyticus</i>	CBS 8294 ^T	AF075525	
<i>Cryptococcus curvatus</i>	CBS 570 ^T	AF189834	
<i>Cryptococcus dimennae</i>	CBS 5770 ^T	AF075489	
<i>Cryptococcus diffluens</i>	CBS 160 ^T	AF075502	AF145330
<i>Cryptococcus diffluens</i> var. <i>uruguiensis</i> ‡	CBS 6436 ^T	AF181543	
<i>Torulopsis albida</i> var. <i>japonica</i> ‡	CBS 926 ^T	AF181542	
<i>Cryptococcus elinovii</i>	CBS 7051 ^T	AF137604	AF145318
<i>Cryptococcus flavus</i>	CBS 331 ^T	AF075497	
<i>Cryptococcus friedmannii</i>	CBS 7160 ^T	AF075478	AF145322
<i>Cryptococcus fuscescens</i>	CBS 7189 ^T	AF075472	AF145319
<i>Cryptococcus gastricus</i>	CBS 2288 ^T	AF137600	
<i>Cryptococcus gastricus</i>	CBS 1927	AF181501	
<i>Cryptococcus gilvescens</i>	CBS 7525 ^T	AF181547	
<i>Cryptococcus heveanensis</i>	CBS 569 ^T	AF075467	
<i>Cryptococcus himalayensis</i>	CBS 6293 ^T	AF181502	
<i>Cryptococcus huempfi</i>	CBS 8186 ^T	AF189844	
<i>Cryptococcus humicolus</i>	CBS 571 ^T	AF189836	
<i>Cryptococcus humicolus</i>	CBS 8354	AF189851	
<i>Cryptococcus humicolus</i>	CBS 8371	AF189854	
<i>Cryptococcus hungaricus</i>	CBS 4214 ^T	AF075503	
<i>Cryptococcus kuetzingii</i>	CBS 1926 ^T	AF137602	AF145327

Table 1 (cont.)

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

Table 1 (cont.)

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

Table 1 (cont.)

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

mycetous 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 genitilis*, *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†
<i>Agaricostilbum hyphaenes</i>	CBS 7811	AF177406	
<i>Bensingtonia ciliata</i>	CBS 7514 ^T	AF189887	
<i>Bensingtonia ingoldii</i>	CBS 7424 ^T	AF189888	
<i>Bensingtonia intermedia</i>	CBS 7226 ^T	AF189889	
<i>Bensingtonia intermedia</i>	CBS 7281	AF189890	
<i>Bensingtonia miscanthi</i>	CBS 7282 ^T	AF189891	
<i>Bensingtonia musae</i>	CBS 7965 ^T	AF189892	
<i>Bensingtonia naganoensis</i>	CBS 7286 ^T	AF189893	
<i>Bensingtonia phyllada</i>	CBS 7169 ^T	AF189894	
<i>Bensingtonia subrosea</i>	CBS 7283 ^T	AF189895	
<i>Bensingtonia yamatoana</i>	CBS 7243 ^T	AF189896	
<i>Bensingtonia yuccicola</i>	CBS 7331 ^T	AF189897	
<i>Chionosphaera apobasidialis</i>	CBS 7430 ^T	AF177407	
<i>Colacogloea peniophorae</i>	IGC 4825	AF189898	
<i>Erythrobasidium hasegavianum</i>	CBS 8253 ^T	AF189899	
<i>Heterogastridium pycnidioideum</i>	CBS 591.93	AF189900	
<i>Kondoa aeria</i>	CBS 8352 ^T	AF189901	
<i>Kondoa aeria</i>	CBS 8378	AF189902	
<i>Kondoa malvinella</i>	CBS 6082 ^T	AF189903	
<i>Kondoa myxariophilla</i>	CBS 8379 ^T	AF189904	
<i>Kriegeria eriophori</i>	CBS 8387	AF189905	
<i>Kurtzmanomyces insolitus</i>	CBS 8377 ^T	AF177408	
<i>Kurtzmanomyces nectairei</i>	CBS 6405 ^T	AF177409	
<i>Kurtzmanomyces tardus</i>	CBS 7421 ^T	AF177410	
<i>Leucosporidium antarcticum</i>	CBS 5942 ^T	AF189906	
<i>Leucosporidium fellii</i>	CBS 7287 ^T	AF189907	
<i>Leucosporidium scottii</i>	CBS 5930 ^T	AF070419	
<i>Leuconostoc scottii</i>	CBS 5932	AF189908	
<i>Occultifur externus</i>	IGC 4817 ^T	AF189909	
<i>Occultifur externus</i>	IGC 4557	AF189910	
<i>Occultifur externus</i>	IGC 4823	AF189911	
<i>Reniforma strues</i>	CBS 8263 ^T	AF189912	
<i>Rhodosporeidium babjevae</i>	CBS 7808 ^T	AF070420	
<i>Rhodosporeidium babjevae</i>	KB 649	AF189913	
<i>Rhodosporeidium diobovatum</i>	CBS 6085 ^T	AF070421	
<i>Rhodosporeidium diobovatum</i>	CBS 6084	AF189914	
<i>Rhodosporeidium fluviale</i>	CBS 6568 ^T	AF070422	
<i>Rhodosporeidium kratochvilovae</i>	CBS 7436 ^T	AF071436	
<i>Rhodosporeidium kratochvilovae</i>	IGC 4818	AF189916	
<i>Rhodosporeidium kratochvilovae</i>	IGC 4819	AF189917	
<i>Rhodosporeidium kratochvilovae</i>	IGC 4793	AF189918	
<i>Rhodosporeidium lusitaniae</i>	CBS 7604 ^T	AF070423	
<i>Rhodosporeidium paludigenum</i>	CBS 6567	AF070424	
<i>Rhodosporeidium sphaerocarpum</i>	CBS 5939 ^T	AF070425	
<i>Rhodosporeidium sphaerocarpum</i>	CBS 5941	AF189919	
<i>Rhodosporeidium toruloides</i>	CBS 349	AF070426	
<i>Rhodotorula araucariae</i>	CBS 6031 ^T	AF070427	
<i>Rhodotorula armeniacae</i>	CBS 8076 ^T	AF189920	
<i>Rhodotorula aurantiaca</i>	CBS 317 ^T	AF189921	
<i>Rhodotorula auriculariae</i>	CBS 6379 ^T	AF189922	
<i>Rhodotorula bogoriensis</i>	CBS 4101 ^T	AF189923	

Table 2 (cont.)

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

Table 2 (cont.)

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

Table 2 (cont.)

Strain	Strain no.*	GenBank accession no.	
		D1/D2	ITS†
<i>Rhodotorula acuta</i> ‡	CBS 7053 ^T	AF189999	
<i>Rhodotorula dulcaminis</i> ‡	CBS 7288 ^T	AF190000	
<i>Sterigmatomyces halophilus</i>	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.
<i>Malassezia furfur</i>	CBS 7019 ^T	AJ249955†
<i>Malassezia globosa</i>	CBS 7966 ^T	AJ249951†
<i>Malassezia obtusa</i>	CBS 7876 ^T	AJ249954†
<i>Malassezia pachydermitis</i>	CBS 1879 ^T	AJ249952†
<i>Malassezia restricta</i>	CBS 7877 ^T	AJ249950†
<i>Malassezia slooffiae</i>	CBS 7956 ^T	AJ249956†
<i>Malassezia sympodialis</i>	CBS 7222 ^T	AJ249953†
<i>Rhodotorula acheniorum</i>	CBS 6386 ^T	AF190001
<i>Rhodotorula bacarum</i>	CBS 6526 ^T	AF190002
<i>Rhodotorula hennulea</i>	CBS 8079 ^T	AF190003
<i>Rhodotorula phylloplana</i>	CBS 8073 ^T	AF190004
<i>Sympodiomyces paphiopedili</i>	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 consortium*). 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 rDNA 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 *Siro-*

basidium 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 al.* 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 Q₉ vel Q₁₀.

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 Q₉ or Q₁₀. **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 montevidense*, *Trichosporon mucoides*, *Trichosporon multisporum*, *Tricho-*

sporon 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 – *Rhodospiridium* 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 *Micro-*

botryum 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 Q₇ (H. J. Roeljmans, personal communication); other yeasts in the Microbotryomycetidae contain coenzyme Q₉ or Q₁₀. 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 *Rhodospiridium* 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 Q₁₀ 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 *Rhodospiridium*; and (2) the *Sporidiobolus ruineniae* branch that includes the ballistoconidia-positive (*Sporidiobolus*) and -negative (*Rhodospiridium*) 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 *Rhodospiridium fluviale*/*Sporidiobolus microsporus* and *Rhodospiridium 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* (*Rhodospiridium*) *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 Q₉, in contrast to the *Sporobolomyces*, *Kurtzmanomyces* branches that exhibit the presence of coenzyme Q₁₀.

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 auricularioid 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 *Sympodiomyces 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. genitilis*) 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. *uruguiensis* 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 zsoitii* (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. zsoitii* 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.

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-

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.

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 oryzae*/ *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.

ACKNOWLEDGEMENTS

This research was supported by the National Science Foundation (Ocean Science Division). David Yarrow provided the CBS strains. The Latin diagnosis was prepared by Walter Gams (CBS). C. P. Kurtzman (USDA, Peoria) provided strains and an insightful review of a draft manuscript. Helen Vishniac (Oklahoma State University) and Brian Steffenson (North Dakota State University) contributed strains. Gina Blatt (University of Miami) furnished technical assistance.

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