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Phylogenetic Relationships among Members of the Ascomycetous Yeast Genera *Brettanomyces*, *Debaryomyces*, *Dekkera*, and *Kluyveromyces* Deduced by Small-Subunit rRNA Gene Sequences

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A molecular systematic investigation of members of the ascomycetous yeast genera *Brettanomyces*, *Debaryomyces*, *Dekkera*, and *Kluyveromyces* was performed by using 18S rRNA gene sequence analysis. Our comparative sequence analysis revealed that *Brettanomyces anomalus* and *Brettanomyces bruxellensis* were closely related to one another and also to their teleomorphs, *Dekkera anomala* and *Dekkera bruxellensis*, respectively. Together with *Dekkera custersiana* and *Dekkera naardenensis*, these four species formed a stable and distinct phylogenetic group. The three representative species of the genus *Debaryomyces* examined (viz., *Debaryomyces castellii*, *Debaryomyces hansenii*, and *Debaryomyces udenii*) were found to be genealogically highly related to each other and exhibited a specific phylogenetic affinity (level of sequence similarity, approximately 99.2%) with *Candida guilliermondii* (teleomorph, *Pichia guilliermondii*). *Debaryomyces* species and *C. guilliermondii* formed a distinct phylogenetic group, which displayed a significant association with a phylogenetically coherent cluster encompassing *Lodderomyces elongisporus*, *Candida albicans*, and four other *Candida* species. In contrast to the situation with the genera *Brettanomyces* and *Debaryomyces*, the genus *Kluyveromyces* displayed very marked phylogenetic heterogeneity. *Kluyveromyces polysporus*, the type species of the genus *Kluyveromyces*, and six other *Kluyveromyces* species (viz., *Kluyveromyces africanus*, *Kluyveromyces delphensis*, *Kluyveromyces lodderae*, *Kluyveromyces thermotolerans*, *Kluyveromyces waltii*, and *Kluyveromyces yarrowii*) were phylogenetically intermixed with species of the genera *Zygosaccharomyces*, *Saccharomyces*, and *Torulaspora*. In contrast, *Kluyveromyces aestuarii*, *Kluyveromyces dobzhanskii*, *Kluyveromyces lactis*, *Kluyveromyces wickerhamii*, and three *Kluyveromyces marxianus* varieties, along with their anamorph, *Candida kefyr*, formed a highly stable monophyletic group worthy of separate generic status. *Kluyveromyces blattae* and *Kluyveromyces phaffii* formed two distinct phylogenetic lines that did not exhibit particularly close affinity with each other or other ascomycetous yeast genera. Our phylogenetic findings are discussed in the context of the results of other genotypic and phenotypic studies.

The yeasts constitute a unique group of fungi that are characterized by vegetative growth that is predominantly unicellular and by the formation of sexual states which are not enclosed in fruiting bodies. The potential phylogenetic diversity of these morphologically simple fungi is illustrated by the fact that some species have ascomycetous life cycles, whereas others have basidiomycetous life cycles; i.e., different yeasts are members of fundamentally different subdivisions of the fungi. In contrast to the situation with bacteria, in which knowledge of evolutionary relationships has been revolutionized by small-subunit rRNA sequence analyses (28), information on the phylogeny of yeasts is limited (12). Although in recent years we have seen an increasing number of phylogenetic investigations involving yeasts (1, 12, 29–31), many of these studies have been based on determinations of relatively small parts (often a few hundred nucleotides) of the small- and/or large-subunit rRNA, an approach which is inherently more prone to error than full sequence analysis (8). The number of known complete (or almost complete) yeast rRNA sequences is still very limited (less than 100 small-subunit rRNA yeast sequences, compared with several thousand bacterial sequences). In order to better understand the evolutionary relationships among the yeasts and to construct more stable and robust phylogenetic frameworks, many more complete rRNA sequences must be determined (6, 8, 27). In this study we used PCR amplification and direct sequencing to determine the almost complete small-subunit rRNA gene sequences of 28 ascomycetous yeast strains belonging to the genera *Brettanomyces*, *Debaryomyces*, *Dekkera*, and *Kluyveromyces*. In this paper the results of a comparative rRNA sequence analysis are presented and inferred evolutionary relationships are discussed in the context of other taxonomic criteria.

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MATERIALS AND METHODS

Yeast strains and cultivation. The yeast strains examined in this study are listed in Table 1. Strains were obtained from the National Collection of Yeast Cultures, Norwich, United Kingdom, and the Centraalbureau voor Schimmelcultures, Delft, The Netherlands. All strains were grown on YM agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 20% agar; pH 5.5) at 25°C.

DNA isolation and purification. To prepare yeast genomic DNA, we used the methods of Sambrook et al. (20) and Philippsen et al. (19), with some modifications. The procedure which we used is described below. Yeast cells were harvested from YM agar plates, resuspended in lysis buffer (1 M sorbitol, 0.1 M EDTA; pH 8.0), and frozen at −70°C. After the cells were thawed at room temperature, 75 μl of a 1,000-U/ml (67-mg/ml) lyticase solution (Sigma) was added to each preparation, and the preparation was incubated at 37°C for 2 h. After 70 μl of a 20% sodium dodecyl sulfate solution (final concentration, 0.3%), 20 μl of a 1-μg/ml RNase A solution, and 30 μl of a 10-μg/ml proteinase K solution were added, the lysis solution was incubated at 37°C for 2 h and extracted three times with phenol-chloroform. DNA was precipitated from the aqueous phase by adding 2 volumes of ice-cold absolute ethanol and 0.1 volume of 3 M sodium acetate (pH 4.8). Pelleted DNA was rinsed with 80% ethanol and dried in air. The DNA was resuspended in 1× TE buffer (pH 8.0), assayed at 260 and 280 nm with a model MPS-2000 Shimadzu spectrophotometer, and diluted for PCR amplification.

TABLE 1. Yeast strains compared in this study and their small-subunit rRNA gene sequence accession numbers

Species or variety	Strain[a]	EMBL and/or GenBank accession no.
Ascosphaera apis	Unknown	M83264
Athelia bombacina	Unknown	M55638
Aureobasidium pullulans	Unknown	M55639
Blastomyces dermatidis	Unknown	M63096
Boletus santanas	TDB-1000	M94337
Brettanomyces anomalus	NCYC 615	X83828[b]
Brettanomyces anomalus	NCYC 749	X83816[b]
Brettanomyces anomalus	NCYC 449^T	X83818[b]
Brettanomyces bruxellensis	NCYC 370^T	X83815[b]
Brettanomyces bruxellensis	NCYC 362	X83814[b]
Bulleromyces albus	MUCL 30301	X60179
Candida albicans	MUCL 29800	X53497
Candida glabrata	CBS 138^T	X51831
Candida guilliermondii	ATCC 6260^T	M60304
Candida holmii	NCYC 137^T	X78601
Candida kefyr (*Candida pseudotropicalis*)	ATCC 4135^T	M60303
Candida krusei	MUCL 29849	M55528
Candida maltosa	IAM 12247	D14593
Candida parapsilosis	ATCC 22019^T	M60307
Candida tropicalis	MUCL 30002	M55527
Candida viswanathii	ATCC 22891^T	M60309
Clavispora lusitaniae	MUCL 29855	M55526
Coprinus cinereus	Unknown	M92991
Cryptococcus neoformans	ATCC 24067	L05428
Cystofilobasidium capitatum	IAM 13521^T	D12801
Debaryomyces castellii	NCYC 604^T	X83819[b]
Debaryomyces hansenii	MUCL 29826	X58053
Debaryomyces udenii	NCYC 2394^T	X83821[b]
Dekkera anomala	CBS 8139^T	X83820[b]
Dekkera bruxellensis	MUCL 27700	X58052
Dekkera custersiana	CBS 4805^T	X83817[b]
Dekkera naardenensis	CBS 6042^T	X85110[b]
Dipodascopsis uninucleata	Unknown	U00969
Endomyces geotrichum	Unknown	U00974
Endomyces fibuliger	MUCL 14481	X69841
Eremascus albus	Unknown	M83258
Filobasidium neoformans	IAM 14211^T	D12804
Galactomyces geotrichum	MUCL 28959	X69842
Glaziella aurantiaca	UME 29396	Z49753
Hanseniaspora uvarum	MUCL 30663	X69844
Kluyveromyces aestuarii	CBS 4438^T	X89520[b]
Kluyveromyces africanus	CBS 2517^T	X89519[b]
Kluyveromyces blattae	CBS 6284^T	X89521[b]
Kluyveromyces cellobiovorus	CBS 7153^T	X89518[b]
Kluyveromyces delphensis	NCYC 768^T	X83823[b]
Kluyveromyces dobzhanskii	NCYC 538^T	X83822[b]
Kluyveromyces lactis	IFO 1267	X51830
Kluyveromyces lodderae	NCYC 1417^T	X83824[b]
Kluyveromyces marxianus var. *bulgaricus*	NCYC 970^T	X89524[b]
Kluyveromyces marxianus var. *marxianus*	CBS 712^T	X89523[b]
Kluyveromyces marxianus var. *wikenii*	CBS 5671^T	X89522[b]
Kluyveromyces phaffii	CBS 4417^T	X89525[b]
Kluyveromyces polysporus	NCYC 523^T	X83825[b]
Kluyveromyces thermotolerans	CBS 6340^T	X89526[b]
Kluyveromyces waltii	CBS 6430^T	X89527[b]
Kluyveromyces wickerhamii	NCYC 546^T	X83826[b]
Kluyveromyces yarrowii	CBS 8242^T	X89528[b]
Lepiota procera	Unknown	L36659
Leucosporidium lari-marini	IAM 14213^T	D12805
Leucosporidium scottii	MUCL 28629	X53499
Lodderomyces elongisporus	CBS 2605^T	X78600
Metschnikowia bicuspidata	MUCL 31145	X69846
Mrakia frigida	IAM 14136^T	D12802
Neolecta vitellina	UME 29192	Z27393
Pichia angusta	ATCC 34438^T	M60310
Pichia anomala	MUCL 28639	X58054
Pichia membranaefaciens	MUCL 30004	X58055
Plectania nigrella	UME 29220	Z27408
Pneumocystis carinii	Unknown	X12708
Rhodotorula glutinis	MUCL 30249	X60180
Rhodotorula graminis	NCYC 502^T	X83827[b]
Rhodotorula mucilaginosa	NCYC 63^T	X84326[b]
Rhodosporidium dacryoidum	IAM 13522	D13459
Rhodosporidium toruloides	IAM 13469^T	D12806
Saccharomyces cerevisiae	Unknown	M27607
Saccharomycodes ludwigii	MUCL 31269	X69843
Saccharomycopsis capsularis	MUCL 27839	X69847
Saitoella complicata	Unknown	D01174
Schizosaccharomyces japonicus var. *versatilis*	Unknown	Z32848
Schizosaccharomyces pombe	Unknown	X54866
Schizophyllum commune	Unknown	X54865
Spongipellis unicolor	Unknown	M59760
Sporidiobolus johnsonii	Unknown	L22261
Sporobolomyces roseus	MUCL 30251^T	X60181
Taphrina deformans	ATCC 34556	U00971
Taphrina populina	CBS 337.55^T	D14165
Taphrina wiesneri	Unknown	D12531
Thanatephorus praticola	Unknown	M92990
Torulaspora delbrueckii	MUCL 27816	X53496
Torulaspora globosa	NCYC 820^T	X84639
Torulaspora pretoriensis	NCYC 524^T	X84638
Trichosporon cutaneum	MUCL 30308[c]	X60182
Urnula hiemalis	UME 30174	Z49754
Ustilago hordei	Unknown	U00973
Ustilago maydis	MUCL 30488	X62396
Waltomyces lipofer	MUCL 27781	X69848
Yarrowia lipolytica	ATCC 18942^T	M60312
Zygosaccharomyces bailii	NCYC 1416^T	X91083
Zygosaccharomyces bisporus	NCYC 1495^T	X91084
Zygosaccharomyces cidri	NCYC 1567^T	X91085
Zygosaccharomyces fermentati	CBS 707^T	X77930
Zygosaccharomyces florentinus	CBS 746^T	X91086
Zygosaccharomyces mellis	NCYC 2403^T	X90755
Zygosaccharomyces microellipsoides	CBS 427^T	X90756
Zygosaccharomyces mrakii	CBS 4218^T	X90757
Zygosaccharomyces rouxii	NCYC 568^T	X90758

[a] NCYC, National Collection of Yeast Cultures, Norwich, United Kingdom; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; ATCC, American Type Culture Collection, Rockville, Md.; MUCL, Mycothèque de l'Université Catholique, Louvain-la-Neuve, Belgium; IAM, Institute of Applied Microbiology, Tokyo, Japan; IFO, Institute of Fermentation, Osaka, Japan.
[b] Sequence determined in this study.
[c] Neotype strain.

PCR amplification and determination of the 18S rRNA gene sequence. The procedure used to amplify 18S rRNA genes was the procedure described by James et al. (8), except that in this study 200 ng of genomic DNA rather than a single colony was used as the PCR template. The three pairs of PCR amplification primers used were P108 plus M2130, P1190 plus M3490, and P2130 plus M3989 (8) (Table 2). After we confirmed that successful amplification had occurred, the PCR products were purified by using a Wizard PCR cleaning kit (Promega, Madison, Wis.) as recommended by the manufacturer. Direct sequencing of the purified PCR products was carried out by using α-^{35}S-labeled dATP and a Sequenase version 2.0 sequencing kit (U.S. Biochemical, Amersham, United Kingdom) as described by Hutson et al. (7). The primers used to determine 18S rRNA gene sequences are shown in Table 2. Both strands of the ribosomal DNA (rDNA) were sequenced.

TABLE 2. Primers used for direct sequencing of small-subunit rRNA genes

Primer	Primer sequence (5′-3′)	Position (*Saccharomyces cerevisiae* numbering)
P108	ACCTGGTTGATCCTGCCAGT	2-21
P130	GTCTCAAAGATTAAGCCATG	34-53
F150	ACTACATGGATA(A/T)C(C/T)GTGC	150-169
R150	CCAC(G/A)G(T/A)TATCCATGTAGT	169-150
M760	CCGTTGAAACCATGGTAG	356-339
P1190	CAATTGGAGGGCAAGTCTGG	543-562
M1190	CCAGACTTGCCCTCCAATTG	562-543
F600	CAGCTCCA(A/G)TAGCGTATA	600-618
R600	TATACGCTA(T/C)TGGAGCTG	618-600
F800	CATGGAATAAT(A/G)GAATAGG	800-819
R800	CCTATTC(T/C)ATTATTCCATG	819-800
P2130	GGTGAAATTCTTGGATTTATTG	900-921
M2130	CAATAAATCCAAGAATTTCACC	921-900
P2540	GGAGTATGGTCGCAAGGCTG	1108-1127
M2540	CAGCCTTGCGACCATACTCC	1127-1108
M3490	TCAGTGTAGCGCGCGTGCGG	1473-1454
M3793	CGACGGGCGGTGTGTACAAAG	1643-1623
FOR3	GCTTAGTGAGGCCTC(C/A)GG	1661-1678
M3989	CTACGGAAACCTTGTTACGACT	1775-1754

Analysis of sequence data. The 18S rRNA gene sequence data were processed by using the Wisconsin Molecular Biology Software Package (2), version 7.2. The 28 18S rDNA sequences which we determined were aligned with the sequences of 88 other ascomycetes and basidiomycetes retrieved from the EMBL data library by using the multiple-sequence alignment program PILEUP (4). The alignments were adjusted manually. An unrooted phylogenetic tree was constructed by using the NEIGHBOR-JOINING program contained in the PHYLIP phylogeny inference package, version 3.5 (3). The confidence values of branches were determined by performing a bootstrap analysis in which 500 replicates were used (3). In addition, a parsimony analysis (3) was performed with a reduced data set comprising species belonging to the subclass Hemiascomycetidae.

Nucleotide sequence accession numbers. The 18S rRNA gene sequences which we determined have been deposited in the EMBL data library under the accession numbers shown in Table 1.

RESULTS AND DISCUSSION

The small-subunit (18S) rRNA gene sequences of 28 yeast strains were amplified in vitro by PCR, and their nucleotide sequences were determined directly. These newly determined sequences were approximately 1,800 nucleotides long and represented more than 99% of the 18S rRNA primary structure. The sequences which we determined were aligned with 88 ascomycetous and basidiomycetous complete or nearly complete 18S rRNA sequences retrieved from the GenBank and EMBL data libraries. Levels of sequence similarity were calculated, and the derived distances were used to infer phylogenetic relationships. Figure 1 is an unrooted tree that was constructed by using the neighbor-joining method and shows the phylogenetic relationships of the yeasts and fungi which we compared. The confidence values for individual branches of the tree were determined by performing a bootstrap analysis in which 500 bootstrap trees were generated from resampled data. The phylogenetic relationships of species belonging to the subclass Hemiascomycetidae were also investigated by performing a parsimony analysis. All of the significant associations (bootstrap values, ≥90% [Fig. 1]) inferred by the neighbor-joining method were confirmed by this method. As shown in Fig. 1, the ascomycetous and basidiomycetous species formed two well-separated lineages. All of the yeast strains sequenced were recovered within subclass Hemiascomycetidae of class Ascomycetes. For a detailed discussion of the subclass Euascomycetidae, see reference 27.

Genera *Brettanomyces* and *Dekkera*. The three *Brettanomyces anomalus* strains examined (NCYC 615, NCYC 749, and NCYC 449^T [T = type strain]) had identical 18S rRNA gene sequences. However, the 18S rRNA gene of *Brettanomyces anomalus* differed at two nucleotide positions from the 18S rRNA gene of its teleomorph, *Dekkera anomala*. Similarly, the 18S rRNA gene sequences of the two *Brettanomyces bruxellensis* strains examined (NCYC 362 and NCYC 370^T) were identical but differed at seven nucleotide positions from the previously published sequence of the teleomorph *Dekkera bruxellensis* (6). Although *Brettanomyces anomalus* (including *Dekkera anomala*) and *Brettanomyces bruxellensis* (including *Dekkera bruxellensis*) are genealogically distinct, it is evident from the level of sequence divergence (approximately 0.1 to 0.4%) and tree resampling measurements (bootstrap value, 100%) that these organisms are phylogenetically closely related species. These findings are in accordance with the results of recent partial small- and large-subunit rRNA sequence (1, 30) and rDNA restriction fragment length polymorphism analyses (18). We found that the strains of *Dekkera custersiana* and *Dekkera naardenensis* which we examined were also specifically, albeit more distantly, related to *Brettanomyces* (*Dekkera*) *anomalus* and *Brettanomyces* (*Dekkera*) *bruxellensis*. Particularly noteworthy were the much lower levels of sequence relatedness (approximately 94.8 to 95.4%) (Table 3) between *Dekkera custersiana* and other *Brettanomyces* (*Dekkera*) species, which is consistent with the data of Yamada et al. (30) and Boekhout et al. (1) based on partial rRNA sequence analyses. Indeed, Yamada et al. (30) concluded that *Dekkera custersiana* should be assigned a teleomorphic genus separate from the genus *Dekkera* van der Walt. Despite the greater level of sequence divergence and somewhat peripheral position of *Dekkera custersiana*, it is evident from the results of our treeing analysis of nearly complete 18S rRNA gene sequences that this species, together with *Brettanomyces* (*Dekkera*) *anomalus*, *Brettanomyces* (*Dekkera*) *bruxellensis*, and *Dekkera naardenensis*, forms a significant phylogenetic group (bootstrap value, 100%). Furthermore, because of the deeper branching position of *Dekkera naardenensis* (Fig. 1), any phylogenetic proposal to assign *Dekkera custersiana* to a separate genus (30) would also mean that a new genus would have to be created for *Dekkera naardenensis*. Although *Eeniella nana* was not included in our analysis, it is pertinent to note that partial 26S rRNA sequencing data (1) indicate that this species also forms a deep and distinct subline within the genus *Brettanomyces*.

Genus *Debaryomyces*. We also determined the phylogenetic relationships of three representative species of the genus *Debaryomyces* (*Debaryomyces castellii*, *Debaryomyces hansenii*, and *Debaryomyces udenii*). These three species were found to be genealogically very closely related (levels of sequence similarity; 99.2 to 99.6%) (Table 4). The close association of these species is consistent with the results of previous studies (13, 31) based on partial small- and large-subunit rRNA sequence data. It was evident from our treeing analysis data that *Candida*

FIG. 1. Dendrogram showing the relationships among species of the genera *Brettanomyces*, *Debaryomyces*, *Dekkera*, and *Kluyveromyces* and related taxa. The tree is based on 18S rRNA gene sequence data and was constructed by using the neighbor-joining method. Bootstrap values were calculated from 500 trees. Organisms whose 18S rRNA sequences were determined in this study are shown in boldface type.

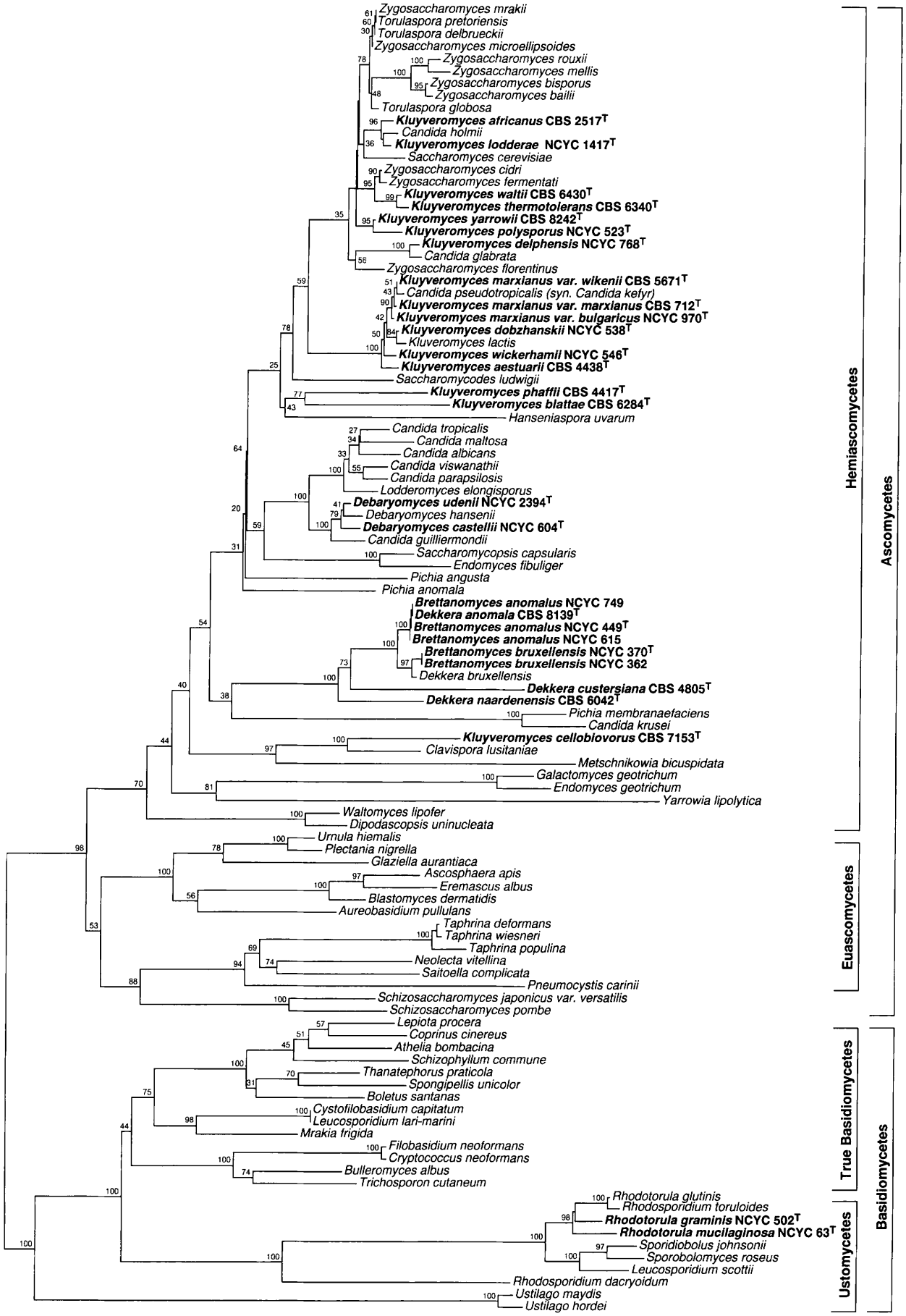
Zygosaccharomyces mrakii
Torulaspora preforiensis
Torulaspora delbrueckii
Zygosaccharomyces microellipsoides
Zygosaccharomyces rouxii
Zygosaccharomyces mellis
Zygosaccharomyces bisporus
Zygosaccharomyces bailii
Torulaspora globosa
Kluyveromyces africanus CBS 2517T
Candida holmii
Kluyveromyces lodderae NCYC 1417T
Saccharomyces cerevisiae
Zygosaccharomyces cidri
Zygosaccharomyces fermentati
Kluyveromyces waltii CBS 6430T
Kluyveromyces thermotolerans CBS 6340T
Kluyveromyces yarrowii CBS 8242T
Kluyveromyces polysporus NCYC 523T
Kluyveromyces delphensis NCYC 768T
Candida glabrata
Zygosaccharomyces florentinus
Kluyveromyces marxianus var. wikenii CBS 5671T
Candida pseudotropicalis (syn. Candida kefyr)
Kluyveromyces marxianus var. marxianus CBS 712T
Kluyveromyces marxianus var. bulgaricus NCYC 970T
Kluyveromyces dobzhanskii NCYC 538T
Kluveromyces lactis
Kluyveromyces wickerhamii NCYC 546T
Kluyveromyces aestuarii CBS 4438T
Saccharomycodes ludwigii
Kluyveromyces phaffii CBS 4417T
Kluyveromyces blattae CBS 6284T
Hanseniaspora uvarum
Candida tropicalis
Candida maltosa
Candida albicans
Candida viswanathii
Candida parapsilosis
Lodderomyces elongisporus
Debaryomyces udenii NCYC 2394T
Debaryomyces hansenii
Debaryomyces castellii NCYC 604T
Candida guilliermondii
Saccharomycopsis capsularis
Endomyces fibuliger
Pichia angusta
Pichia anomala
Brettanomyces anomalus NCYC 749
Dekkera anomala CBS 8139T
Brettanomyces anomalus NCYC 449T
Brettanomyces anomalus NCYC 615
Brettanomyces bruxellensis NCYC 370T
Brettanomyces bruxellensis NCYC 362
Dekkera bruxellensis
Dekkera custersiana CBS 4805T
Dekkera naardenensis CBS 6042T
Pichia membranaefaciens
Candida krusei
Kluyveromyces cellobiovorus CBS 7153T
Clavispora lusitaniae
Metschnikowia bicuspidata
Galactomyces geotrichum
Endomyces geotrichum
Yarrowia lipolytica
Waltomyces lipofer
Dipodascopsis uninucleata
Urnula hiemalis
Plectania nigrella
Glaziella aurantiaca
Ascosphaera apis
Eremascus albus
Blastomyces dermatidis
Aureobasidium pullulans
Taphrina deformans
Taphrina wiesneri
Taphrina populina
Neolecta vitellina
Saitoella complicata
Pneumocystis carinii
Schizosaccharomyces japonicus var. versatilis
Schizosaccharomyces pombe
Lepiota procera
Coprinus cinereus
Athelia bombacina
Schizophyllum commune
Thanatephorus praticola
Spongipellis unicolor
Boletus santanas
Cystofilobasidium capitatum
Leucosporidium lari-marini
Mrakia frigida
Filobasidium neoformans
Cryptococcus neoformans
Bulleromyces albus
Trichosporon cutaneum
Rhodotorula glutinis
Rhodosporidium toruloides
Rhodotorula graminis NCYC 502T
Rhodotorula mucilaginosa NCYC 63T
Sporidiobolus johnsonii
Sporobolomyces roseus
Leucosporidium scottii
Rhodosporidium dacryoidum
Ustilago maydis
Ustilago hordei
Hemiascomycetes
Euascomycetes
True Basidiomycetes
Ustomycetes
Ascomycetes
Basidiomycetes

TABLE 3. Levels of 18S rRNA gene sequence similarity for *Brettanomyces* and *Dekkera* strains

Organism	% 18S rRNA sequence similarity									
	Brettanomyces anomalus NCYC 749	*Brettanomyces anomalus* NCYC 449^T	*Brettanomyces anomalus* NCYC 615	*Brettanomyces bruxellensis* NCYC 370^T	*Brettanomyces bruxellensis* NCYC 362	*Dekkera anomala* CBS 8139^T	*Dekkera bruxellensis* MUCL 27700	*Dekkera custersiana* CBS 4805^T	*Dekkera naardenensis* CBS 6042^T	*Saccharomyces cerevisiae*
Brettanomyces anomalus NCYC 749	100	100	100	99.5	99.5	99.9	99.2	95.4	96.6	93.4
Brettanomyces anomalus NCYC 449^T		100	100	99.5	99.5	99.9	99.2	95.0	96.6	93.5
Brettanomyces anomalus NCYC 615			100	99.5	99.5	99.9	99.2	95.3	96.6	93.4
Brettanomyces bruxellensis NCYC 370^T				100	100	99.4	99.6	94.8	96.4	93.6
Brettanomyces bruxellensis NCYC 362					100	99.4	99.6	95.4	96.3	93.6
Dekkera anomala CBS 8139^T						100	99.3	95.1	96.5	93.3
Dekkera bruxellensis MUCL 27700							100	95.1	96.4	93.4
Dekkera custersiana CBS 4805^T								100	95.1	91.7
Dekkera naardenensis CBS 6042^T									100	93.4
Saccharomyces cerevisiae										100

guilliermondii (teleomorph, *Pichia guilliermondii*) is a close phylogenetic relative (levels of sequence similarity; 99 to 99.2%; bootstrap value, 100%) of these *Debaryomyces* species. Five *Candida* species (*Candida albicans*, *Candida maltosa*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida viswanathii*) and *Lodderomyces elongisporus* formed a phylogenetically separate, but nevertheless highly significant, association (intergroup levels of sequence similarity, approximately 96.6 to 98.1%; bootstrap value, 100%) with the *Debaryomyces-Candida guilliermondii* cluster.

Genus *Kluyveromyces*. It was evident from the results of the phylogenetic analysis that the genus *Kluyveromyces* is not a monophyletic group and that several species are intermixed with members of other ascomycetous genera (Fig. 1). A total of 14 of the *Kluyveromyces* strains compared fell into two major phylogenetic clusters, whereas 3 strains formed relatively distinct lines. Cluster 1 comprised nutritionally restricted species, some of which produce more than four spores per ascus (*Kluyveromyces africanus*, *Kluyveromyces delphensis*, *Kluyveromyces lodderae*, *Kluyveromyces polysporus*, and *Kluyveromyces yarrowii*) (15, 25), the high-G+C-content species *Kluyveromyces thermotolerans* and *Kluyveromyces waltii* (5), and species belonging to the genera *Saccharomyces*, *Torulaspora*, and *Zygosaccharomyces*. Within this group several very significant phylogenetic associations between *Kluyveromyces* species were apparent. For example, consistent with the results of an rDNA restriction analysis (21), *K. polysporus* and *K. yarrowii* were found to be genealogically closely related (level of sequence similarity, 99.4%), as were *K. africanus* and *K. lodderae* (level of sequence similarity, 99.5%) (Table 5). We also found that *Candida holmii* (teleomorph, *Saccharomyces exiguus*) has a close phylogenetic affinity (levels of sequence similarity, 99.1 to 99.3%) with the latter two organisms. Similarly, we found that *K. thermotolerans* and *K. waltii* are highly related (level of sequence similarity, 99.7%). The close phylogenetic affinity between the last two species correlates well with the results of

TABLE 4. Levels of 18S rRNA gene sequence similarity for *Debaryomyces* species and their close relatives

Organism	% 18S rRNA gene sequence similarity										
	Candida albicans MUCL 29800	*Candida guilliermondii* ATCC 6260^T	*Candida maltosa* IAM 12247	*Candida parapsilosis* ATCC 22019^T	*Candida tropicalis* MUCL 30002	*Candida viswanathii* ATCC 22891^T	*Debaryomyces castellii* NCYC 604^T	*Debaryomyces hansenii* MUCL 29826	*Debaryomyces udenii* NCYC 2394^T	*Lodderomyces elongisporus* CBS 2605^T	*Saccharomyces cerevisiae*
Candida albicans MUCL 29800	100	96.1	97.4	98.3	98.5	98.8	96.6	96.6	96.6	97.7	95.2
Candida guilliermondii ATCC 6260^T		100	96.3	97.7	97.0	98.2	99.0	99.2	99.2	96.7	95.4
Candida maltosa IAM 12247			100	97.9	97.9	98.6	97.0	96.8	97.1	97.1	93.8
Candida parapsilosis ATCC 22019^T				100	99.0	99.3	98.0	98.0	97.9	98.7	95.5
Candida tropicalis MUCL 30002					100	99.5	97.8	97.7	98.1	98.7	95.3
Candida viswanathii ATCC 22891^T						100	98.0	98.1	98.0	98.6	95.5
Debaryomyces castellii NCYC 604^T							100	99.2	99.6	97.8	95.2
Debaryomyces hansenii MUCL 29826								100	99.4	97.6	95.1
Debaryomyces udenii NCYC 2394^T									100	97.8	95.5
Lodderomyces elongisporus CBS 2605^T										100	95.2
Saccharomyces cerevisiae											100

TABLE 5. Levels of 18S rRNA gene sequence similarity for representative *Kluyveromyces*, *Torulaspora*, and *Zygosaccharomyces* strains and related organisms

Organism	% 18S rRNA gene sequence similarity																														
	Candida glabrata CBS 138^T	*Candida holmii* NCYC 137^T	*Candida pseudotropicalis* ATCC 4135^T	*Kluyveromyces aestuarii* CBS 4438^T	*Kluyveromyces africanus* CBS 2517^T	*Kluyveromyces blattae* CBS 6284^T	*Kluyveromyces delphensis* NCYC 768^T	*Kluyveromyces dobzhanskii* NCYC 538^T	*Kluyveromyces lactis* IFO 1267	*Kluyveromyces lodderae* NCYC 1417^T	*Kluyveromyces marxianus* var. *bulgaricus* NCYC 970^T	*Kluyveromyces marxianus* var. *marxianus* CBS 712^T	*Kluyveromyces marxianus* var. *wikenii* CBS 5671^T	*Kluyveromyces phaffii* CBS 4417^T	*Kluyveromyces polysporus* NCYC 523^T	*Kluyveromyces thermotolerans* CBS 6340^T	*Kluyveromyces waltii* CBS 6430^T	*Kluyveromyces wickerhamii* NCYC 546^T	*Kluyveromyces yarrowii* CBS 8242^T	*Saccharomyces cerevisiae*	*Saccharomycodes ludwigii* MUCL 31269	*Torulaspora delbrueckii* MUCL 27816^T	*Torulaspora globosa* NCYC 820^T	*Zygosaccharomyces bailii* NCYC 1416^T	*Zygosaccharomyces cidri* NCYC 1567^T	*Zygosaccharomyces fermentati* CBS 707^T	*Zygosaccharomyces florentinus* CBS 746^T	*Zygosaccharomyces mellis* NCYC 2403^T	*Zygosaccharomyces microellipsoides* CBS 427^T	*Zygosaccharomyces mrakii* CBS 4218^T	*Zygosaccharomyces rouxii* NCYC 568^T
Candida glabrata CBS 138^T	100	98.1	97.0	96.3	98.4	96.2	99.5	96.8	96.8	98.1	96.8	96.5	96.8	96.2	97.6	96.7	96.8	96.8	98.2	97.6	97.0	98.5	98.2	97.3	97.4	97.3	98.4	97.2	98.4	98.4	97.5
Candida holmii NCYC 137^T		100	97.1	96.5	99.1	96.1	98.2	96.9	96.8	99.3	96.9	97.3	97.0	96.3	98.5	98.2	98.4	97.1	98.9	98.6	97.3	98.9	98.8	97.9	98.6	98.5	98.7	97.6	98.9	98.9	97.9
Candida pseudotropicalis ATCC 4135^T			100	99.4	97.7	95.0	96.9	99.5	99.4	97.2	99.7	99.7	99.8	95.3	95.7	97.4	97.4	99.5	97.9	96.9	96.5	97.6	97.4	96.7	97.7	97.5	97.4	96.5	97.5	97.4	96.8
Kluyveromyces aestuarii CBS 4438^T				100	97.2	94.9	96.5	99.4	99.2	96.8	99.5	99.5	99.6	95.5	97.0	97.1	97.2	99.5	97.6	96.3	96.0	97.1	97.2	96.3	97.5	97.5	97.1	96.1	97.2	97.1	96.3
Kluyveromyces africanus CBS 2517^T					100	96.1	98.4	97.7	97.4	99.5	97.7	97.5	97.8	96.4	98.5	97.8	98.0	97.7	98.9	98.6	97.2	99.0	99.0	97.9	98.1	98.1	98.6	97.8	99.2	99.1	98.1
Kluyveromyces blattae CBS 6284^T						100	96.4	95.2	94.9	96.2	95.1	94.8	95.2	96.0	95.4	95.3	95.3	95.2	95.8	95.0	94.6	95.8	96.0	95.3	95.0	95.2	96.5	95.2	95.8	95.7	95.3
Kluyveromyces delphensis NCYC 768^T							100	97.0	96.7	98.3	97.0	96.7	97.1	96.3	97.9	97.0	97.1	97.1	98.3	97.7	96.9	98.4	98.4	97.5	97.6	97.5	98.6	97.4	98.5	98.4	97.6
Kluyveromyces dobzhanskii NCYC 538^T								100	99.6	97.3	99.7	99.7	99.7	95.5	97.2	97.4	97.4	99.6	97.9	96.7	96.5	97.4	97.3	96.4	97.7	97.6	97.3	96.3	97.5	97.4	96.6
Kluyveromyces lactis IFO 1267									100	97.0	99.4	99.4	99.4	95.1	96.8	97.2	97.2	99.3	97.6	96.5	96.3	97.4	97.0	96.1	97.3	97.2	97.0	95.8	97.1	97.1	96.2
Kluyveromyces lodderae NCYC 1417^T										100	97.3	97.6	97.3	96.2	98.5	98.3	98.4	97.3	98.8	98.6	97.0	99.1	99.0	97.9	98.6	98.5	98.5	97.8	99.2	99.2	98.0
Kluyveromyces marxianus var. *bulgaricus* NCYC 970^T											100	99.9	99.9	95.6	97.3	97.4	97.4	99.6	98.0	96.8	96.5	97.6	97.4	96.4	97.7	97.6	97.3	96.2	97.6	97.5	96.5
Kluyveromyces marxianus var. *marxianus* CBS 712^T												100	99.9	95.6	97.1	97.5	97.5	99.6	97.7	97.1	96.5	97.3	97.2	96.4	97.7	97.6	97.1	96.2	97.3	97.3	96.1
Kluyveromyces marxianus var. *wikenii* CBS 5671^T													100	95.6	97.3	97.5	97.5	99.7	98.1	96.8	96.6	97.6	97.5	96.5	97.7	97.6	97.4	96.2	97.6	97.6	96.5
Kluyveromyces phaffii CBS 4417^T														100	96.3	96.0	96.0	95.4	97.0	96.0	94.4	96.3	96.2	95.0	95.8	95.7	96.2	95.8	96.1	96.2	95.0
Kluyveromyces polysporus NCYC 523^T															100	97.7	97.9	97.1	99.4	98.4	96.8	98.9	97.4	96.6	96.5	96.4	96.9	96.3	97.5	97.4	96.5
Kluyveromyces thermotolerans CBS 6340^T																100	99.7	97.4	98.1	98.0	97.0	98.2	98.1	97.8	99.3	99.1	97.3	97.4	98.3	98.2	97.6
Kluyveromyces waltii CBS 6430^T																	100	97.6	98.3	98.1	97.1	98.3	98.3	97.9	99.6	99.4	97.6	97.6	98.5	98.4	97.9
Kluyveromyces wickerhamii NCYC 546^T																		100	97.8	96.6	96.5	97.5	97.4	96.5	97.8	97.7	97.5	96.3	97.6	97.5	96.6
Kluyveromyces yarrowii CBS 8242^T																			100	98.8	97.6	99.3	99.3	98.2	98.4	98.3	98.7	98.0	99.4	99.3	98.3
Saccharomyces cerevisiae																				100	96.8	98.8	98.6	97.4	98.3	98.2	98.0	97.2	98.8	98.8	97.5
Saccharomycodes ludwigii MUCL 31269																					100	97.4	97.1	96.3	96.8	96.7	96.9	96.0	97.2	97.1	96.3
Torulaspora delbrueckii MUCL 27816^T																						100	99.7	98.4	98.5	98.4	98.9	98.3	99.8	99.8	98.7
Torulaspora globosa NCYC 820^T																							100	99.0	98.5	98.5	99.0	98.8	99.8	99.7	98.9
Zygosaccharomyces bailii NCYC 1416^T																								100	98.4	98.4	98.4	99.0	98.9	98.8	99.3
Zygosaccharomyces cidri NCYC 1567^T																									100	99.8	98.1	98.0	98.7	98.5	98.2
Zygosaccharomyces fermentati CBS 707^T																										100	98.1	98.0	98.6	98.4	98.2
Zygosaccharomyces florentinus CBS 746^T																											100	98.1	99.1	98.9	98.3
Zygosaccharomyces mellis NCYC 2403^T																												100	98.7	98.7	99.5
Zygosaccharomyces microellipsoides CBS 427^T																													100	99.9	98.9
Zygosaccharomyces mrakii CBS 4218^T																														100	98.8
Zygosaccharomyces rouxii NCYC 568^T																															100

phenotypic and genotypic studies (5, 11), including rDNA restriction analyses (14, 21). Interestingly, *Zygosaccharomyces cidri* and *Zygosaccharomyces fermentati* also exhibited a close (levels of sequence similarity, approximately 99.1 to 99.6%) and significant (bootstrap value, >90%) association with *K. thermotolerans* and *K. waltii*. *K. delphensis* did not exhibit a particularly close phylogenetic affinity with any other *Kluyveromyces* species but did exhibit a highly significant association with *Candida glabrata* (level of sequence similarity, 99.5% bootstrap value, 100%). *K. delphensis* and *Candida glabrata* share many characteristics, including similar assimilation and fermentation profiles, coenzyme Q compositions, and genomic G+C contents (17, 25). It is clear from the treeing analysis results that the seven *Kluyveromyces* species in cluster 1, including *K. polysporus*, the type species of the genus *Kluyveromyces*, are phylogenetically intermixed with members of the genera *Saccharomyces*, *Torulaspora*, and *Zygosaccharomyces*. The overall levels of 18S rRNA sequence similarity for the cluster 1 species are very high (generally >97%). Although there are many significant species associations, as well as considerable internal structure, in this phylogenetic group, it is clear from the branching pattern of the tree that no separation of the genera *Kluyveromyces*, *Saccharomyces*, *Torulaspora*, and *Zygosaccharomyces* along traditional lines is possible phylogenetically.

Cluster 2 comprised five *Kluyveromyces* species (*Kluyveromyces aestuarii*, *Kluyveromyces dobzhanskii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus* [including *K. marxianus* var. *marxianus*, *K. marxianus* var. *bulgaricus*, and *K. marxianus* var. *wikenii*], and *Kluyveromyces wickerhamii*) and *Candida pseudotropicalis* (Fig. 1). The members of cluster 2 formed a highly related (levels of intragroup sequence similarity, >99%) and significant (bootstrap value, 100%) group that was well separated from the cluster 1 organisms and other *Kluyveromyces* species. These findings are consistent with the results of an rDNA restriction analysis (21). The ability of *K. dobzhanskii*, *K. lactis*, *K. marxianus*, and *K. wickerhamii* to mate in the laboratory (9, 10, 24) is also consistent with the close affinity of these species. The anamorph-teleomorph relationship between *Candida pseudotropicalis* (synonym, *Candida kefyr*) and *K. marxianus* varieties has been demonstrated by DNA reassociation analysis (5) and isoenzyme analysis (22), although there is evidence of heterogeneity in these species (23, 26). However, it is absolutely clear from our data that the members of cluster 2 form a phylogenetically coherent group worthy of a distinct genus.

Kluyveromyces blattae and *Kluyveromyces phaffii* formed distinct lines of descent that were quite separate from each other and from the cluster 1 and 2 species (Fig. 1). The loose association of *K. blattae*, *K. phaffii*, *Hanseniaspora uvarum*, and other species is not statistically significant, as shown by the relatively low bootstrap values (Fig. 1). The recovery of *K. blattae* as a distinct line is consistent with evidence obtained from mating studies (9, 24), DNA reassociation analysis (5), and rDNA restriction mapping (14, 21), all of which showed that *K. blattae* is a distantly related species of the genus *Kluyveromyces*. Using rDNA restriction analysis, Shen et al. (21) recovered *K. phaffii* in the same group as *K. thermotolerans* and *K. waltii*. These authors (21) noted that the position of *K. phaffii* was somewhat uncertain and that this species may occupy an intermediate position between *K. africanus* and *K. thermotolerans*. The results of our analysis of almost complete 18S rRNA gene sequences, however, clearly show that *K. phaffii* is an independent line that is far removed from *K. africanus*, *K. thermotolerans*, and *K. waltii*, all of which are members of phylogenetic cluster 1 (Fig. 1). Irrespective of the taxonomic complexities associated with the intermixing of many *Kluyveromyces* species with other ascomycetous genera (e.g., the genus *Zygosaccharomyces*), it is evident from the sequence divergence values and the results of the treeing analysis that both *K. blattae* and *K. phaffii* should be excluded from the genus *Kluyveromyces* and warrant new separate genera.

Kluyveromyces cellobiovorus CBS 7153^T was also found to be phylogenetically far removed from other *Kluyveromyces* species (Fig. 1). This finding is consistent with the results of an rDNA restriction analysis (21) in which *K. cellobiovorus* formed a line quite separate from other members of the genus *Kluyveromyces*. *K. cellobiovorus*, which is not known to form ascospores, is conspecific with *Candida intermedia*. *K. cellobiovorus* and *Candida intermedia* exhibit very high levels of nuclear DNA homology (>90%) (16). It is evident from the results of our investigation that *K. cellobiovorus* should be excluded from the genus *Kluyveromyces*. Furthermore, our data show that *K. cellobiovorus* exhibits a highly significant, albeit distant, phylogenetic association with *Clavispora lusitaniae* (level of sequence similarity, 96.5%; bootstrap value, 100%).

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