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Identification of Clinically Important Ascomycetous Yeasts Based on Nucleotide Divergence in the 5′ End of the Large-Subunit (26S) Ribosomal DNA Gene

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Clinically important species of *Candida* and related organisms were compared for extent of nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA (rDNA) gene. This rDNA region is sufficiently variable to allow reliable separation of all known clinically significant yeast species. Of the 204 described species examined, 21 appeared to be synonyms of previously described organisms. Phylogenetic relationships among the species are presented.

Yeasts, including those of clinical importance, are often defined based on cellular morphology and distinctive reactions in a standardized set of fermentation and assimilation tests. However, genetic crosses, as well as molecular comparisons, have shown that many of these phenotypic characteristics are strain specific and inadequate for recognition of either species or genera (12, 15). The use of genetic crosses to routinely define species is also problematical. Results from crosses between compatible mating types of heterothallic species are sometimes difficult to interpret, and this is especially true for attempts to cross homothallic organisms (11). Given these difficulties, and the widespread occurrence of asexual species, systematists have turned increasingly to molecular comparisons for definition and identification of yeast species. Kurtzman (9) and Kurtzman and Phaff (12) reviewed results from nuclear DNA (nDNA) reassociation studies of various heterothallic ascomycetous yeasts and noted that members of a biological species generally exhibit 70% or greater nDNA complementarity. Isolates with 40 to 70% nDNA relatedness are often considered varieties of the same species unless genetic crosses indicate otherwise. These criteria have been applied to homothallic as well as to anamorphic species with the argument that strains of these groups appear to have neither greater nor lesser ranges of DNA relatedness than those found among heterothallic species.

Disadvantages of nDNA reassociation studies include the need for pairwise comparisons of all species under study and the fact that resolution is limited to the genetic distance of sibling species. Consequently, interest has turned to molecular sequencing, with the expectation that both close and distant relationships can be resolved by comparing appropriate molecules. Peterson and Kurtzman (23) examined the nucleotide sequence of the variable D2 region near the 5′ end of large-subunit (LSU) (26S) rRNA and demonstrated resolution of various heterothallic sibling species in the genera *Issatchenkia*, *Pichia*, and *Saccharomyces*. Their work showed that conspecific strains generally had fewer than 1% nucleotide substitutions in region D2, whereas separate biological species had greater than this number, thus providing an empirical means for recognizing species.

Molecular comparisons are leading to an understanding of phylogenetic relationships among yeasts (2, 7, 13, 33) and offer the opportunity for rapid, unambiguous identification of clinical isolates. In the latter context, various investigators have proposed identification of *Candida* species based on analysis of restriction fragment length polymorphisms of ribosomal DNA (rDNA) (18–21, 32) and on oligonucleotide-based molecular probes that hybridize with species-specific RNA or DNA sequences (4, 5, 30). However, relatively few species have been compared, and successful application of molecular methods for strain identification requires prior characterization of all known species in the phylogenetic group of interest as well as an understanding of the extent of intraspecific nucleotide divergence.

In the present study, sequences were determined for the ca. 600-nucleotide variable region (D1/D2) at the 5′ end of the LSU rDNA gene for all currently recognized species of *Candida*. Phylogenetically defined groups that include many of the clinically important *Candida* species were then compared with related ascosporic species. The rDNA region examined in this study was shown earlier to be sufficiently variable to recognize nearly all species assigned to *Saccharomyces*, *Williopsis*, and selected sections of *Pichia* (11, 13, 23). We have further tested this observation by comparing nDNA complementarity with rDNA nucleotide divergence for closely related representative species included in the present study. Additionally, multiple strains were examined for some species, such as *Candida albicans*, to assess intraspecific variation.

MATERIALS AND METHODS

Organisms and culture conditions. The strains studied are listed in Table 1, and all are maintained in the Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Ill. Cells used for DNA extraction were grown for approximately 24 h at 25°C in 50 ml of Wickerham's (31) YM broth (3 g of yeast extract, 3 g of malt extract, 5 g of peptone, and 10 g of glucose per liter of distilled water) on a rotary shaker at 200 rpm and harvested by centrifugation. The cells were washed once with distilled water and resuspended in 2 ml of distilled water, and 1 ml of the suspension was pipetted into a microcentrifuge tube. After centrifugation, excess water was decanted from the microcentrifuge tube and the packed cells were lyophilized for 1 to 2 days and stored in a freezer (−20°C) until use.

DNA isolation, PCR, and sequencing reactions. DNA isolation for PCR was performed by a modified version of the sodium dodecyl sulfate protocol of Raeder and Broda (25). The lyophilized cell mass was broken apart in a 1.5-ml microcentrifuge tube with a pipette tip, and ca. 0.5 ml of 0.5-mm-diameter glass

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TABLE 1. Strains of *Candida* and reference species compared

Organism	Strain designation[a] NRRL	CBS	No. of nucleotide differences between type strains and conspecific isolates	GenBank accession no.[b]
Aciculoconidium aculeatum	YB-4298[A]	2282		U40087*
Arxula adeninivorans	Y-17692	8244		U40094*
	Y-17693[A]	7370	2	
A. terrestris	Y-17704	7376		U40103*
Ascoidea africana	Y-17632	377.68		U40131*
Blastobotrys arbuscula	Y-17585	227.83		U40108*
B. aristata	Y-17579	521.75		U40109*
B. capitulata	Y-17573	287.82		U40104*
B. elegans	Y-17572	530.83		U40095*
B. nivea	Y-17581	163.67		U40110*
B. proliferans	Y-17577	522.75		U40098*
Candida aaseri	YB-3897	1913		U45802
C. albicans	Y-12983	562		U45776
	Y-17967[A]		1	
	Y-17968[A]		1	
	Y-17974[A]		1	
	Y-17976[A]		2	
	YB-3898[A]	1912	1	
C. ancudensis	Y-17327	8184		U45810
C. antillancae	Y-17673	8170		U45812
C. apicola	Y-2481	2868		U45703
C. apis	Y-2482	2674		U48237
C. atlantica	Y-17759	5263		U45799
C. atmosphaerica	Y-17642	4547		U45779
C. auringiensis	Y-17674	6913		U62300
C. austromarina	Y-17769	6179		U62310
	Y-17852[A]	6588	0	
C. azyma	Y-17067	6826		U62312
C. beechii	Y-17758	4261		U45798
C. berthetii	Y-17644	5452		U62298
C. blankii	Y-17068	1898		U45704
C. boleticola	Y-17080	6420		U45777
C. bombi	Y-17081	5836		U45706
C. bombicola	Y-17069	6009		U45705
C. bondarzewiae	Y-17328	8171		U45806
C. buinensis	Y-11706	6796		U45778
C. butyri	Y-17648	6421		U45780
C. cacaoi	Y-17649	2020		U45744
C. cantarellii	Y-17650	4878		U45814
C. castrensis	Y-17329	8172		U45807
C. catenulata	Y-1508	565		U45714
C. chilensis	Y-7790	5719		U45821
C. chiropterorum	Y-17071	6064		U45822
C. coipomoensis	Y-17651	8178		U45747
C. conglobata	Y-1504	2018		U45789
C. cylindracea	Y-17506	6330		U45823
C. dendrica	Y-7775	6151		U62301
C. dendronema	Y-7781	6270		U45751
C. diddensiae	Y-7589	2214		U45750
C. drimydis	Y-17675	8185		U45815
C. dublinensis[c]	Y-17841	7987		U57685
	Y-17512[A]		0	
	Y-17969[A]		0	
	Y-17971[A]		0	
	Y-17972[A]		0	
	Y-17973[A]		0	
	Y-17975[A]		1	
C. entomaea	Y-7785	6306		U45790
C. entomophila	Y-7783	6160		U62302
C. ergastensis	Y-17652	6248		U45746
C. etchellsii	Y-17084	1750		U45723
C. fennica	Y-7505	5928		U45715
C. fermenticarens	Y-17321	7040		U45756
C. floricola	Y-17676	7289		U45710
C. fluviatilis	Y-7711	6776		U45717
C. friedrichii	Y-17653	4114		U45781
C. fructus	Y-17072	6380		U44810
C. fukuyamaensis	Y-17857	7921		U62311
C. galacta	Y-17645	6939		U45820
C. geochares	Y-17073	6870		U48591
C. glabrata	Y-65	138		U44808
C. glaebosa	Y-6949	5691		U45757
C. glucosophila	Y-17781	7349		U45849
C. gropengiesseri	Y-1445	156		U45721
C. haemulonii	Y-6693	5149		U44812
	Y-17799[A]	5150	0	
	Y-17800[A]	7801	0	
C. haemulonii type II	Y-17801	6915		U44819
	Y-17802[A]	7798	0	
C. halonitratophila	Y-6694	5240		U45725
C. halophila	Y-2483	4019		U45828
C. homilentoma	Y-10941	6312		U45716
C. incommunis	Y-17085	5604		U62303
C. insectalens	Y-7778	6036		U62304
C. insectamans	Y-7786	6033		U45753
C. insectorum	Y-7787	6213		U45791
C. intermedia	Y-981	572		U44809
C. krissii	Y-17086	6519		U45853
C. kruisii	Y-17087	6451		U45718
C. lactis-condensi	Y-1515	52		U45724
	Y-2338[A]	2633	0	
C. laureliae	Y-17656	8180		U45787
C. lodderae	Y-17317	1924		U45755
C. lyxosophila	Y-17539	8194		
	Y-17538[A]	7268	0	U45801
"*C. magnifica*"[d]	Y-5717			U44821
C. magnoliae	Y-2024	166		U45722
C. maltosa	Y-17677	5611		U45745
C. mannitofaciens	Y-7226	5981		U45830
C. melibiosica	Y-17076	5814		U44813
C. membranifaciens	Y-2089	1952		U45792
C. mesenterica	Y-1494	602		U45720
C. mogii	Y-17032	2032		U44820
C. montana	Y-17326	8057		U62305
C. multigemmis	Y-17659	6524		U45782
C. musae	Y-17088	6381		U44814
C. naeodendra	Y-10942	6032		U45759
C. natalensis	Y-17680	2935		U45818
C. nodaensis	Y-2484	3094		U45726
C. norvegica	Y-17660	4239		U62299
C. oleophila	Y-2317	2219		U45793
C. oregonensis	Y-5850	5036		U44815
C. palmioleophila	Y-17323	7418		U45758
C. paludigena	Y-12697	8005		U45826
C. parapsilosis	Y-12969	604		U45754
	Y-543[A]		0	
C. pararugosa	Y-17089	1010		U62306
C. petrohuensis	Y-17663	8173		U45819
C. pseudointermedia	Y-10939	6918		U44816
C. psychrophila	Y-17665	5956		U45813
C. quercitrusa	Y-5392	4412		U45831
	Y-5704[A]		1	

Continued on following page

TABLE 1—*Continued*

Organism	Strain designation[a] NRRL	Strain designation[a] CBS	No. of nucleotide differences between type strains and conspecific isolates	GenBank accession no.[b]
C. railenensis	Y-17762	8164		U45800
C. ralunensis	Y-17666	8179		U45786
C. rhagii	Y-2594	4237		U45729
C. rugosa	Y-95	613		U45727
C. saitoana	Y-17316	940		U45762
C. sake	Y-1622	159		U45728
	Y-1499[A]	617	0	
C. salmanticensis	Y-17090	5121		U62308
C. santamariae var. *santamariae*	Y-6656	4515		U45794
C. santamariae var. *membranifaciens*	Y-17647	5838		U45785
C. santjacobensis	Y-17667	8183		U45811
C. savonica	Y-17077	6563		U62307
C. schatavii	Y-17078	6452		U45795
C. sequanensis	Y-17682	8118		U45711
C. shehatae var. *shehatae*	Y-12858	5813		U45761
C. shehatae var. *insectosa*	Y-12854	4286		U45773
C. shehatae var. *lignosa*	Y-12856	4705		U45772
C. sophiae-reginae	Y-17668	8175		U45817
C. sorbophila	Y-7921	6739		U45852
C. sorboxylosa	Y-17669	6378		U62314
C. spandovensis	Y-17761	6875		U62309
C. stellata	Y-1446	157		U45730
C. suecica	Y-12943	5724		U45732
C. tanzawaensis	Y-17324	7422		U44811
C. tenuis	Y-1498	615		U45774
C. tepae	Y-17670	5115		U45816
C. terebra	Y-17683	6023		U45784
C. torresii	Y-6699	5152		U45731
C. tropicalis	Y-12968	94		U45749
	Y-5716[A]	4913	0	
	Y-1552[A]	433	0	
C. tsuchiyae	Y-17840	7195		U49064
C. vaccinii	Y-17684	7318		U45708
C. valdiviana	Y-7791	5721		U45835
C. vanderwaltii	Y-17671	5524		U62313
C. veronae	Y-17672	5815		U45783
C. versatilis	Y-6652	1752		U45834
C. vinaria	Y-5715	4077		U45833
C. viswanathii	Y-6660	4024		U45752
C. xestobii	Y-17685	5975		U45707
C. zeylanoides	Y-1774	619		U45832
Candida sp.	Y-17456			U45775
Cephaloascus fragrans	Y-6742	121.29		U40091*
Clavispora lusitaniae	Y-11827	6936		U44817
C. opuntiae	Y-11820	7068		U44818
Debaryomyces carsonii	YB-4275	2285		U45743
D. castellii	Y-7423	2923		U45841
D. coudertii	Y-7425	5167		U45846
D. etchellsii	Y-7121	2011		U45809
D. hansenii	Y-7426	767		U45808
D. marama	Y-2171	1958		U45838
D. melissophilus	Y-7585	6344		U45740
D. nepalensis	Y-7108	5921		U45839
D. occidentalis var. *occidentalis*	Y-10	819		U45804
D. occidentalis var. *persoonii*	Y-7400	2169		U45840
D. polymorphus	Y-2022	186		U45836
D. pseudopolymorphus	YB-4229	2008		U45845
D. robertsiae	Y-6670	2934		U45805
D. udenii	Y-17354	7056		U45844
D. vanrijiae var. *vanrijiae*	Y-7430	3024		U45842
D. vanrijiae var. *yarrowii*	Y-7535	6246		U45843
D. yamadae	Y-11714	7035		U45837
Debaryomyces sp.	Y-7804			U45771
Dekkera bruxellensis	Y-12961	74		U45738
Dipodascopsis uninucleata	Y-17583	190.37		U40137*
Galactomyces geotrichum	Y-17569	772.71		U40118*
Lipomyces starkeyi	Y-11557	1807		U45824
Lodderomyces elongisporus	YB-4239	2605		U45763
	Y-7681[A]	5912	0	
Mastigomyces philippovii	Y-17708	7047		U45760
Metschnikowia australis	Y-17414	5847		
	Y-7014[A]	5848	0	U44824
M. bicuspidata	YB-4993	5575		U44822
M. gruessii	Y-17809	7657		U45737
M. hawaiiensis	Y-17272	7432		U45734
M. krissii	Y-5389	4823		U45735
M. lunata	Y-7131	5946		U45733
M. pulcherrima	Y-7111	5833		U45736
M. reukaufii	Y-7112	5834		U44825
M. zobellii	Y-5387	4821		U44823
Nadsonia fulvescens	Y-12810	2596		U45825
	Y-991[A]		1	
Pichia abadieae	Y-7499	6067		U46123
P. acaciae	Y-7117	5656		U45767
P. burtonii	Y-1933	2352		U45712
P. castillae	Y-7501	6053		U45769
P. farinosa	Y-7553	185		U45739
	Y-12695[A]	7064	0	
P. guilliermondii	Y-2075	2030		U45709
	Y-2076[A]	2031	0	
	Y-17818[A]		0	
	Y-17819[A]		0	
	Y-17820[A]		0	
	Y-17970[A]		1	
P. haplophila	Y-7860	2028		U45770
P. heimii	Y-7502	6139		U45713
P. inositovora	Y-12698	8006		U45848
P. media	Y-7122	5521		U45768
P. mexicana	Y-11818	7066		U45797
P. nakazawae var. *nakazawae*	Y-7903	6700		U45748
P. nakazawae var. *akitaensis*	Y-7904	6701		U45766
P. ofunaensis	Y-10998	8129		U45829
P. ohmeri	Y-1932	5367		U45702
	Y-2078[A]	2037	0	
P. philogaea	Y-7813	6696		U45765
P. scolyti	Y-5512	4802		U45788
P. segobiensis	Y-11571	6857		U45742
P. spartinae	Y-7322	6059		U45764
P. stipitis	Y-7124	5773		U45741

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TABLE 1—*Continued*

Organism	Strain designation[a] NRRL	Strain designation[a] CBS	No. of nucleotide differences between type strains and conspecific isolates	GenBank accession no.[b]
P. tannicola	Y-17392	6065		U45803
P. triangularis	Y-5714	4094		U45796
Saccharomyces cerevisiae	Y-12632	1171		U44806
Schizosaccharomyces pombe	Y-12796	356		U40085*
Sporopachydermia lactativora	Y-11591	6192		U45851
Stephanoascus ciferrii	Y-10943	5295		U40138*
S. farinosus	Y-17593	140.71		U40132*
Sympodiomyces parvus	Y-10004	6147		U40096*
Trigonopsis variabilis	Y-1579	1040		U45827
Wickerhamia fluorescens	YB-4819	4565		U45719
Wickerhamiella domercqiae	Y-6692	4351		U45847
Yarrowia lipolytica	YB-423	6124		U40080*
	Y-1095[A]	6317	0	
Zygoascus hellenicus	Y-17319[A]	4099		U40125*
	Y-17346	7521	0	
Zygozyma oligophaga	Y-17247	7107		U45850

[a] All are type strains except those designated as authentic (superscript A). NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research. CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands.
[b] *, sequences published previously by Kurtzman and Robnett (15). All other sequences are new to this study.
[c] The original spelling of the species epithet for *C. dublinensis* as "*dubliniensis*" (26) has been treated as an orthographic error.
[d] "*Candida magnifica*" was originally received from O. Verona and apparently was never validly described.

beads was added to the microcentrifuge tube. The tube was shaken for 20 min on a wrist action shaker at maximum speed. This treatment visibly fractured about 25% of the cells. The cells were suspended in 1 ml of extraction buffer (200 mM Tris-HCl [pH 8.5], 250 mM NaCl, 25 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate) and extracted with phenol-chloroform and chloroform. DNA was precipitated from the aqueous phase by adding 0.54 volume of isopropanol and pelleted for ca. 3 min in an Eppendorf model 5415 microcentrifuge at 14,000 rpm. The pellet was washed gently with 70% ethanol, resuspended in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and dissolved by incubation at 55°C for 1 to 2 h. Dilute DNA samples for PCR were prepared by adding 4 μl of the genomic stocks to 1 ml of 0.1× TE buffer.

The divergent domain at the 5′ end of the LSU rDNA gene (6) was symmetrically amplified with primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5′-GGTCCGTGTTTCAAGACGG) (22). Amplification was performed for 36 PCR cycles with annealing at 52°C, extension at 72°C for 2 min, and denaturation at 94°C for 1 min. The amplified DNA was purified with Geneclean II (Bio 101, La Jolla, Calif.) according to the manufacturer's instructions. Visualization of the amplified DNA was performed following Geneclean II treatment by electrophoresis in 1.5% agarose in 1× TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA [pH 8.0]) and staining with ethidium bromide (8×10^{-5} μg/μl).

Both strands of the rDNA regions compared were sequenced with the ABI TaqDyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems Inc., Foster City, Calif.). Four sequencing reactions were required for each DNA sample. Primers for these reactions were the external primers NL-1 and NL-4 and the internal primers NL-2A (5′-CTTGTTCGCTATCGGTCTC) and NL-3A (5′-GAGACCGATAGCGAACAAG).

Sequence data were visually aligned with QEdit 2.15 (SemWare, Marietta, Ga.). Phylogenetic relationships were calculated with a Power Macintosh 8500/120 by the maximum-parsimony program of PAUP 3.1.1 (28) with a heuristic search employing both simple and random sequence additions. Relationships were further analyzed by the neighbor-joining program of PAUP 4.0d47 (27a) with the Jukes-Cantor distance measure. *Schizosaccharomyces pombe* was the designated outgroup in all analyses. Confidence limits for phylogenetic trees were estimated from bootstrap analyses (100 replications for heuristic searches and 1,000 for neighbor-joining searches). The aligned data set used for calculating the phylogenetic trees presented in Fig. 1 and 2 is available as a computer file from us.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited with GenBank under the accession numbers shown in Table 1.

TABLE 2. nDNA relatedness and LSU rDNA region D1/D2 nucleotide divergence between type strains of closely related yeasts

Strain comparison group[a]	nDNA reassociation (%)[b]	No. of rDNA nucleotide differences
Candida etchellsii		
'*C. halonitratophila*'	84	1
'*Candida nodaensis*'	89	0
C. shehatae var. *shehatae*		
C. shehatae var. *insectosa*	49	1
C. shehatae var. *lignosa*	46	0
C. shehatae var. *insectosa*		
C. shehatae var. *lignosa*	59	1
C. versatilis		
'*C. halophila*'	83	1
'*C. mannitofaciens*'	91	2
Debaryomyces hansenii		
D. coudertii	16	4
D. marama	8	7
D. nepalensis	15	6
D. robertsiae	6	8
D. melissophilus		
Debaryomyces sp.	32	2
D. occidentalis var. *occidentalis*		
D. occidentalis var. *persoonii*	83	2
D. polymorphus		
D. castellii	20	5
D. pseudopolymorphus	21	3
D. vanrijiae var. *vanrijiae*	24	8
D. vanrijiae var. *yarrowii*		7
D. vanrijiae var. *vanrijiae*		
D. vanrijiae var. *yarrowii*	68	1
Pichia nakazawae var. *nakazawae*		
P. nakazawae var. *akitaensis*	41	2
P. segobiensis		
P. stipitis	38	1
P. tannicola		
'*P. abadieae*'	100	0

[a] Organism names in single quotation marks are synonyms of the first organism listed in each group.
[b] nDNA reassociation data are from Kurtzman (8, 10), Kurtzman and Robnett (reference 14 and unpublished data), Price et al. (24), and Suzuki et al. (27).

RESULTS

Resolution of species. An initial question in this work was whether the species under study could be identified based on divergence in LSU rDNA region D1/D2. Comparisons in Ta-

TABLE 3. Matrix of nucleotide differences in LSU rDNA region D1/D2 between organisms of the *C. albicans* clade

Organism	No. of nucleotide differences[a]								
	C. parapsilosis	*Candida* sp.	*C. tropicalis*	*C. maltosa*	*C. viswanathii*	*C. lodderae*	*C. albicans*	*C. dublinensis*	*L. elongisporus*
Candida sp.	6								
C. tropicalis	26	23							
C. maltosa	31	27	17						
C. viswanathii	43	37	21	32					
C. lodderae	43	37	22	32	1				
C. albicans	45	43	39	43	33	33			
C. dublinensis	43	42	35	39	37	37	13		
L. elongisporus	19	19	25	34	41	42	49	46	
C. lyxosophila	55	51	54	58	55	55	49	48	57

[a] Nucleotide difference determinations were based on comparisons of type strains.

ble 1 show that intraspecific variations range from 0 to 2 nucleotides. The results for *C. albicans*, *Candida dublinensis*, and *Pichia guilliermondii* may be more predictive than those for the other species because the analyses included additional (six to seven) strains. Comparisons in Table 2 substantiate the observation that members of a species show no more than 0 to 2 nucleotide substitutions. However, these data also demonstrate that strains with as little as 40% nDNA complementarity can be indistinguishable from one another by sequence differences in region D1/D2. For example, *Candida shehatae* var. *shehatae* and *Candida shehatae* var. *lignosa*, with 46% nDNA relatedness, have identical sequences. Nonetheless, known clinically important species are more divergent from one another than the varieties just discussed and can be reliably separated by sequence determinations. For example, nucleotide differences among members of the *C. albicans* clade range from 6 to 49 (Table 3).

Synonymous taxa. A prediction based on the sequence comparisons in this study is that 21 of the species examined are synonyms of previously described species (Table 4). Diagnostic physiological reactions for most of the synonyms are similar to those of their basionyms. One notable exception concerns *Candida sake* and *Candida austromarina*. Barnett et al. (1) reported *C. sake* to be more fermentative, to assimilate a larger number of carbon compounds, and to have an optimum growth temperature at least 5°C greater than *C. austromarina*. In view of these differences, sequences were determined for one additional strain of each species as well as for replacement cultures of the type strains. The sequences for all six cultures were identical, thus demonstrating that phenotypic variability can be quite marked among isolates of some species.

Phylogeny. The present data set (182 taxa with 626 characters, of which 405 are parsimony informative) contains insufficient phylogenetic information to provide strong statistical support for deep lineages, but our goal was to examine close relationships and to determine the usefulness of the D1/D2 region for recognition of individual species. The phylogenetic trees presented in Fig. 1 and 2 were calculated by neighbor joining with the Jukes-Cantor distance measure. Trees determined by maximum parsimony were congruent with those determined by neighbor joining except for the following: *Candida insectamans* arose on a long branch from within the *Pichia stipitis* clade, and *P. guilliermondii* was found on a branch adjacent to *Candida glucosophila.*

The phylogenetic analyses presented in Fig. 1 and 2 show that pathogenic species are not unique to a particular clade. For example, *Pichia* (*Candida*) *guilliermondii* and *Candida zeylanoides* are not closely related to one another or to the *C. albicans* clade which includes the recently described *C. dublinensis* (26) as well as *Candida viswanathii* and its apparent synonym *Candida lodderae* (Table 3); *Candida tropicalis*, *Candida parapsilosis*, and a closely related new species, *Candida maltosa*; and the ascosporic species *Lodderomyces elongisporus* (Fig. 1). *Candida haemulonii* and a new species tentatively described as *C. haemulonii* type II (17) are a sister group to the *Clavispora* (*Candida*) *lusitaniae* clade, which in turn is a sister group to the *Metschnikowia* clade (Fig. 2). Most of the species included in this study are characterized by production of an ubiquinone (coenzyme Q) with a side chain of nine isoprene units (1). Our analysis shows that this particular ubiquinone occurs in a broad assemblage of species.

DISCUSSION

Although yeast species are often defined based on cellular morphology and on distinctive reactions in a standardized set of fermentation and assimilation tests, genetic crosses as well as molecular comparisons have shown that many of these phenotypic characteristics are unreliable for defining and recognizing species (12, 15). Additionally, application of the biological species concept to yeasts and other fungi is often not practical because of the difficulty in obtaining genetic crosses and assessing their outcome (29). Use of the phylogenetic-species concept, especially when based on cladistic analysis of molecular characteristics, offers constancy in the circumscription of species and gives a more realistic appraisal of biodiversity.

Peterson and Kurtzman (23) correlated the biological species concept with the phylogenetic species concept through comparison of the fertility of genetic crosses among divergent heterothallic ascomycetous yeasts with the extent of nucleotide substitutions in the variable LSU rDNA region D2. Their study demonstrated that strains of biological species generally show fewer than 1% substitutions in region D2. One exception concerned *Saccharomyces bayanus* and *Saccharomyces pastorianus*. Although the two species had the same D2 sequence, it appears that *S. pastorianus* arose as a hybrid of *S. bayanus* and *S. cerevisiae* and received the rDNA genes of *S. bayanus*, which have not yet undergone divergence. In the present work, we have observed that certain taxonomic varieties and sibling species (Table 2) show no greater number of nucleotide substitutions than are found between highly related strains of a species. Consequently, verification of predicted relatedness between strains showing few nucleotide differences will need to be made based on nDNA reassociation or on sequence differences in a gene with a higher rate of evolution than that of region D1/D2. Nonetheless, it appears to be possible to accurately identify most yeast species based on their nucleotide sequence in LSU

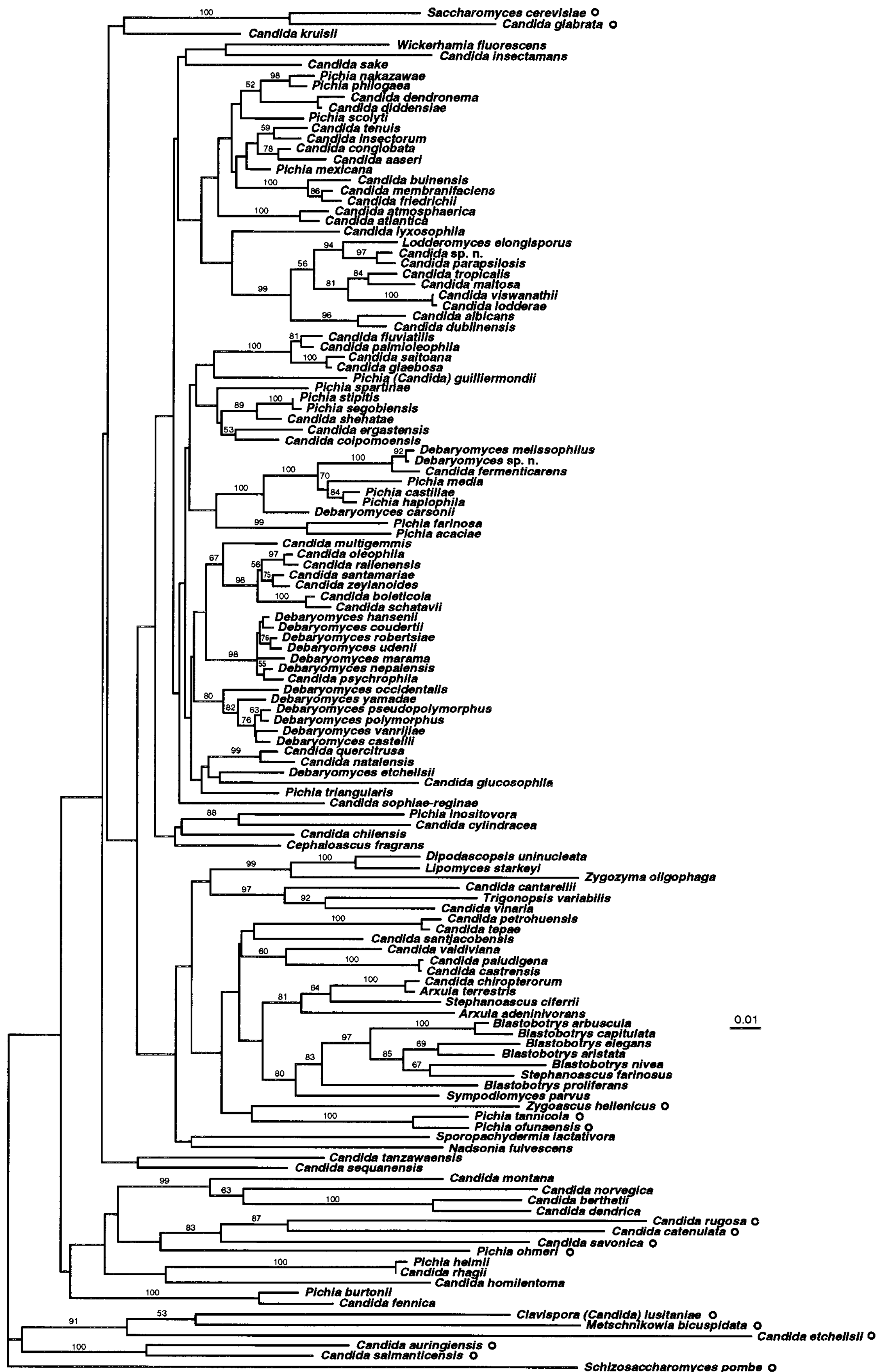

FIG. 1. Phylogenetic tree derived from neighbor-joining analysis depicting members of the *C. albicans*, *Debaryomyces*, and *Stephanoascus* clades and various reference species. The phylogram was calculated based on nucleotide divergence in the 5′ end of the LSU rDNA gene (positions 63 to 680). Branch lengths are proportional to the number of nucleotide differences, and the numbers given on branches are the frequencies (percentages) with which a given branch appeared in 1,000 bootstrap replications. Values for frequencies of less than 50% are not given. The marker bar denotes relative branch lengths. Organism names followed by a circle are also shown in Fig. 2.

TABLE 4. Predicted relatedness among described species of *Candida* and other genera with similar or identical nucleotide sequences in LSU rDNA region D1/D2

Organism comparison group[a]	No. of rDNA nucleotide differences	Predicted relatedness
C. aaseri		
C. butyri	0	Same species
C. boleticola		
C. laureliae	1	Same species
C. ralunensis	1	Same species
C. diddensiae		
C. naeodendra	0	Same species
C. fructus		
C. musae	0	Same species
C. paludigena		
C. castrensis	2	Same or sib species
C. petrohuensis		
C. ancudensis	0	Same species
C. drimydis	0	Same species
C. sake		
C. austromarina	0	Same species
C. santamariae var. *santamariae*		
C. beechii	0	Same species
C. santamariae var. *membranifaciens*	2	Same or sib species
C. tenuis		
Mastigomyces philoppovii	1	Same species
C. tepae		
C. antillancae	0	Same species
C. bondarzewiae	0	Same species
C. viswanathii		
C. lodderae	1	Same species
C. zeylanoides		
C. krissii	0	Same species
Metschnikowia reukaufii		
"*C. magnifica*"	2	Same or sib species
Pichia farinosa		
C. cacaoi	0	Same species
Pichia (*Candida*) *guilliermondii*		
C. fukuyamaensis	1	Same species
C. xestobii	1	Same species
P. heimii		
C. rhagii	2	Same or sib species
P. mexicana		
C. veronae[b]	0	Same species

[a] Comparisons were made with the type strains of the species listed, and organisms became synonyms if conspecific with the first organism listed in each group.

[b] Lee et al. (16) demonstrated on the basis of nDNA relatedness that *C. veronae*, *C. entomaea*, and *C. terebra* are conspecific; all have identical D1/D2 sequences.

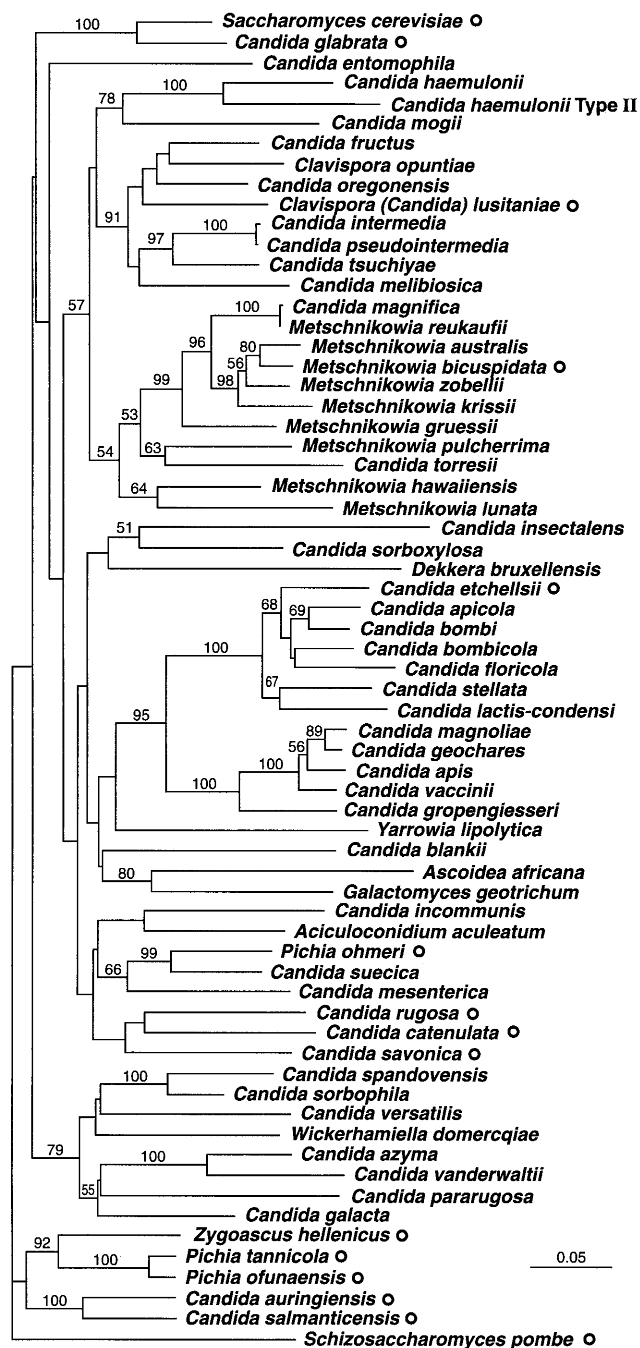

FIG. 2. Phylogenetic tree derived from neighbor-joining analysis depicting members of the *Metschnikowia*/*Candida etchellsii* clade. Other pertinent information is given in the legend to Fig. 1. Organism names followed by a circle are also shown in Fig. 1.

rDNA region D1/D2, and this procedure can be quite rapid when done with an automated DNA sequencer.

The phylogeny of selected pathogenic *Candida* species has been examined by Barns et al. (2) and by Wilmotte et al. (33), who showed from comparisons of small-subunit (18S) rRNA sequences that *C. albicans*, *C. viswanathii*, *C. tropicalis*, and *C. parapsilosis* were closely related and that members of this clade were progressively less related to *C. guilliermondii*, *C. lusitaniae*, *Candida krusei*, and *Candida glabrata*, respectively. In-

clusion of many additional species in the present study has given a more precise phylogenetic placement of most medically important species. Earlier studies placed *C. glabrata* near *S. cerevisiae* (2, 33). In a comparison of small-subunit rDNA sequences from nearly all described species of the *Saccharomyces*/*Kluyveromyces* clade, Cai et al. (3) showed *C. glabrata* to be closely related to *Kluyveromyces delphensis.*

The phylogenetic analyses presented in Fig. 1 and 2 show that many presently accepted genera are poorly circumscribed and will require redescription in the future. The polyphyly of *Candida* (2, 33) and of *Pichia* (23) was apparent from earlier studies. Prior work (13, 14) suggested *Debaryomyces* to be monophyletic, but with the inclusion of species of *Candida* and *Pichia* in the analysis, *Debaryomyces* now appears to be polyphyletic. Among anamorphs included in Fig. 1, species of *Arxula*, *Blastobotrys*, and *Sympodiomyces* appear to be congeneric. Before taxonomic changes can be made, however, additional sequence data will be required to develop a more statistically robust analysis.

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