JOURNAL OF CLINICAL MICROBIOLOGY, June 1999, p. 1985–1993 Vol. 37, No. 6
0095-1137/99/$04.00+0

Identification of Medically Relevant *Trichosporon* Species Based on Sequences of Internal Transcribed Spacer Regions and Construction of a Database for *Trichosporon* Identification

TAKASHI SUGITA,[1] AKEMI NISHIKAWA,[2] REIKO IKEDA,[1] AND TAKAKO SHINODA[1]*

Department of Microbiology[1] and Department of Immunobiology,[2] Meiji Pharmaceutical University, Kiyose, Tokyo, Japan

Received 16 October 1998/Returned for modification 14 January 1999/Accepted 1 March 1999

The nucleotide sequences of the internal transcribed spacer (ITS) 1 and 2 regions in the rRNA gene were determined by directly sequencing PCR-amplified fragments for all of the species (17 species and five varieties) in the genus *Trichosporon*. Comparative sequence analysis suggests that six medically relevant species, *T. asahii*, *T. asteroides*, *T. cutaneum*, *T. inkin*, *T. mucoides*, and *T. ovoides*, can be readily identified by their ITS sequences. In addition, the sequence analysis showed that conspecific strains have fewer than 1% nucleotide differences in the ITS 1 and 2 regions overall. Molecular phylogenetic trees are also presented.

Trichosporon Behrend is a medically important genus that includes the causative agents of both deep-seated, mucosa-associated infections and superficial infections, including white piedra (8, 28, 51). The majority of leukemia and lymphoma patients with fatal disseminated fungemia are in a profound neutropenic state when their infections develop. Recently, the number of patients with illness caused by *Trichosporon* species has been increasing (10, 14, 43, 44, 48, 50). Deep-seated trichosporonosis has a high mortality rate, and the prognosis for patients is very poor. *Trichosporon* species are also responsible for summer-type hypersensitivity pneumonitis (1, 2, 33, 49).

In 1992, the taxonomy of the genus *Trichosporon* was significantly revised by Guého et al. (5). Subsequently, Sugita et al. (37, 42) proposed a new classification that included new species, and 17 species and five varieties are presently accepted in the genus. Recent taxonomic studies indicate that trichosporonosis is caused by six species: *T. asahii*, *T. asteroides*, *T. cutaneum*, *T. inkin*, *T. mucoides*, and *T. ovoides* (4, 7, 40, 41). Moreover, it has been suggested that the major causative agents of trichosporonosis differ in each type of infection. *T. asahii* and *T. mucoides* are involved in deep-seated infection. *T. asteroides* and *T. cutaneum* are associated with superficial infection. *T. ovoides* and *T. inkin* are involved in white piedra of the head and genital area, respectively. *T. pullulans* is not a major causative agent of trichosporonosis and is rarely isolated from fungemia patients (16, 17).

On the other hand, at least four different serological types (I, II, III, and I-III) of *Trichosporon* have been identified in species by Ikeda et al. (9) and Nishiura et al. (30). The six medically relevant species are serotype I or II. Serotypes III and I-III do not seem to be responsible for infection.

Although we have developed a PCR-based identification system with genus-specific and *T. asahii* species-specific primers (38, 39), rapid identification of all *Trichosporon* species is not yet possible.

In this study, we sequenced the internal transcribed spacer (ITS) regions for all of the species in the genus *Trichosporon* and developed an identification system for all of the species.

MATERIALS AND METHODS

Strains used. The strains used in this study are shown in Table 1. They included stock strains and clinical and environmental isolates. *Trichosporon* sp. strain M 9481 was isolated from the soil in Tokyo, Japan. *Candida albicans*, *Candida famata*, *Debaryomyces hansenii*, and *Saccharomyces cerevisiae* were also used.

Direct DNA sequencing. Nuclear DNA was extracted by the method of Makimura et al. (22). ITSs 1 and 2 and the intervening 5.8S ribosomal DNA (rDNA) region were amplified with primers pITS-F (GTCGTAACAAGGTTA ACCTGCGG) and pITS-R (TCCTCCGCTTATTGATATGC), which were designed from conserved regions of the 18S and 28S rRNA genes, respectively. The reactions were performed in a final reaction mixture (50 μl) containing 10 pmol of each primer; 200 mM (each) dATP, dTTP, dGTP, and dCTP; 2.5 mM $MgCl_2$; 0.5 U of *Ex Taq* polymerase (Takara, Shiga, Japan); and 5 μl of 10× reaction buffer (Takara). The amplification reactions were performed in a GeneAmp PCR System 9700 (Perkin-Elmer Applied Biosystems, Foster City, Calif.) with the following cycling parameters: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 10 min. The amplified products were purified with a NucleoSpin DNA purification kit (Macherey-Nagel GmbH, Duren, Germany) according to the manufacturer's instructions. Direct sequencing of the PCR product was performed with a PRISM Cycle sequencing kit (Perkin-Elmer Applied Biosystems). Two external primers, pITS-F and pITS-R, were used to determine the sequences.

Nucleotide sequence similarity. The similarities of the sequences were compared by using the nuclear DNA relatedness values taken from the literature (11–13, 18, 19, 24–27, 29, 36, 37, 40–42) and the similarity of the ITS 1 and 2 sequences separately. Sequence similarity was visually determined from pairwise alignments. The nucleotide sequences of species other than those in the genus *Trichosporon* included in this study were obtained from GenBank, and their accession numbers are cited in Table 1.

Molecular phylogenetic analysis. The sequences were aligned with the computer program CLUSTAL W (45). For the neighbor-joining analysis (32), the distances between the sequences were calculated by using Kimura's two-parameter model (15). Sites where gaps existed in any of the sequences were excluded. The program DNAML in PHYLIP version 3.5c was used for the maximum-likelihood analysis (3). *T. pullulans* was used as the outgroup.

Identification of ubiquinone. The ubiquinone study was carried out only with the *Trichosporon* sp. strain M 9481 environmental isolate. The extraction of ubiquinone was performed following a procedure of Yamada and Kondo (47) with a slight modification. Ubiquinone was isolated by thin-layer chromatography (50 by 200 mm) (PK6F silica gel; Whatman, Clifton City, N.J.) in hexane-diethyl ether (85:15 [vol/vol]) and detected with UV light (254 nm). The type of ubiquinone was determined by high-performance liquid chromatography under the following conditions: reverse-phase column, Zorbax ODS (150 by 4.6 mm; Shimadzu, Kyoto, Japan); mobile phase, ethanol-water (97:3 [vol/vol]); column

* Corresponding author. Mailing address: Department of Microbiology, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo, 204-8588 Japan. Phone: 81-424-95-8761. Fax: 81-424-95-8761. E-mail: shinoda@my-pharm.ac.jp.

TABLE 1. Strains used and accession numbers of ITS sequences

Species	Strain	Source[a]	Accession no.
Trichosporon aquatile	M 9317^T	CBS 5973	AB018011 (this study)
Trichosporon aquatile	M 9321	CBS 5988	AB018012 (this study)
Trichosporon aquatile	M 9472	Environmental isolate	AB018012 (this study)
Trichosporon aquatile	M 9473	Environmental isolate	AB018011 (this study)
Trichosporon asahii var. *asahii*	M 9306^T	CBS 2479	AB018013 (this study)
Trichosporon asahii var. *asahii*	M 9311	CBS 2530	AB018014 (this study)
Trichosporon asahii var. *asahii*	M 9470	Clinical isolate	AB018013 (this study)
Trichosporon asahii var. *asahii*	M 9474	Clinical isolate	AB018014 (this study)
Trichosporon asahii var. *asahii*	M 9475	Clinical isolate	AB018013 (this study)
Trichosporon asahii var. *asahii*	M 9476	Environmental isolate	AB018013 (this study)
Trichosporon asahii var. *asahii*	M 9477	Environmental isolate	AB018013 (this study)
Trichosporon asahii var. *coremiformis*	M 9309^T	CBS 2482	AB018015 (this study)
Trichosporon asahii var. *faecalis*	M 9312^T	CBS 4828	AB018016 (this study)
Trichosporon asteroides	M 9308^T	CBS 2481	AB018017 (this study)
Trichosporon asteroides	M 9329	CBS 7623	AB018018 (this study)
Trichosporon asteroides	M 9330	CBS 7624	AB018017 (this study)
Trichosporon brassicae	M 9322^T	CBS 6382	AB018019 (this study)
Trichosporon cutaneum	M 9304^T	CBS 2466	AB018020 (this study)
Trichosporon cutaneum	M 9307	CBS 2480	AB018020 (this study)
Trichosporon domesticum	M 9401	Environmental isolate (42)	AB018021 (this study)
Trichosporon domesticum	M 9412	Clinical isolate	AB018021 (this study)
Trichosporon dulcitum	M 9337^T	CBS 8257	AB018022 (this study)
Trichosporon dulcitum	M 9318	CBS 5785	AB018022 (this study)
Trichosporon gracile	M 9334^T	CBS 8189	AB018023 (this study)
Trichosporon gracile	M 9335	CBS 8193	AB018023 (this study)
Trichosporon inkin	M 9316^T	CBS 5585	AB018024 (this study)
Trichosporon inkin	M 9333	CBS 7629	AB018024 (this study)
Trichosporon jirovecii	M 9325^T	CBS 6864	AB018025 (this study)
Trichosporon jirovecii	M 9326	CBS 6950	AB018026 (this study)
Trichosporon loubieri var. *loubieri*	M 9327^T	CBS 7065	AB018027 (this study)
Trichosporon loubieri var. *laibachii*	M 9319^T	CBS 5790	AB018028 (this study)
Trichosporon moniliiforme	M 9305^T	CBS 2467	AB018029 (this study)
Trichosporon montevideense	M 9323^T	CBS 6721	AB018021 (this study)
Trichosporon montevideense	M 9338	CBS 8261	AB018021 (this study)
Trichosporon mucoides	M 9331^T	CBS 7625	AB018030 (this study)
Trichosporon mucoides	M 9478	Environmental isolate	AB018031 (this study)
Trichosporon mucoides	M 9479	Environmental isolate	AB018031 (this study)
Trichosporon mucoides	M 9480	Environmental isolate	AB018031 (this study)
Trichosporon mucoides	M 9422	Clinical isolate (41)	AB018030 (this study)
Trichosporon ovoides	M 9315	CBS 5580	AB018033 (this study)
Trichosporon ovoides	M 9328^T	CBS 7556	AB018032 (this study)
Trichosporon ovoides	M 9458	Environmental isolate (36)	AB018032 (this study)
Trichosporon pullulans	M 9339^T	CBS 2532	AB018034 (this study)
Trichosporon sporotrichoides	M 9336^T	CBS 8245	AB018035 (this study)
Trichosporon sp.	M 9481	Environmental isolate	AB018036 (this study)
Candida albicans	M 1001^T	CBS 562	AB018037 (this study)
Candida albicans	M 1016	ATCC 10264	AB018038 (this study)
Candida albicans	M 1445	NIHB 792	AB018037 (this study)
Candida albicans	M 1447	NIHA 207	AB018037 (this study)
Candida albicans	M 1601	CBS 1905	AB018038 (this study)
Candida albicans	M 1602	CBS 1918	AB018038 (this study)
Candida albicans	M 2088	IFO 1061	AB018037 (this study)
Candida albicans	M 2089	IFO 1389	AB018037 (this study)
Candida albicans	M 2091	IFO 0583	AB018037 (this study)
Candida albicans	M 2093	IFO 0579	AB018037 (this study)
Candida famata var. *famata*	M 5033^T	JCM 1521	AB018039 (this study)
Candida famata var. *flarei*	M 5024^T	JCM 2166	AB018040 (this study)
Debaryomyces hansenii var. *hansenii*	M 5012^T	JCM 1990	AB018041 (this study)
Debaryomyces hansenii var. *hansenii*	M 5112	Clinical isolate (29)	AB018041 (this study)
Debaryomyces hansenii var. *fabryi*	M 5011^T	JCM 2104	AB018042 (this study)
Debaryomyces hansenii var. *fabryi*	M 5102	Clinical isolate (29)	AB018042 (this study)
Saccharomyces cerevisiae	M 6013^T	CBS 1171	AB018043 (this study)
Candida parapsilosis	MCO 429		U10989
Candida parapsilosis	MCO 448		U10989
Saccharomyces bayanus	CBS 380^T		D89887
Saccharomyces bayanus	CBS 395		Z95946
Saccharomyces bayanus	CBS 425		Z95944

Continued on following page

TABLE 1—*Continued*

Species	Strain	Source[a]	Accession no.
Saccharomyces bayanus	CBS 1546		Z95948
Saccharomyces cerevisiae	CBS 382		Z95936
Saccharomyces cerevisiae	CBS 400		Z95939
Saccharomyces cerevisiae	CBS 423		Z95932
Saccharomyces cerevisiae	CBS 2247		Z95937
Saccharomyces cerevisiae	CBS 3081		Z95941
Saccharomyces cerevisiae	CBS 3093		Z95943
Saccharomyces cerevisiae	CBS 4903		Z95940
Saccharomyces cerevisiae	CBS 5378		Z95929
Saccharomyces cerevisiae	CBS 5635		Z95942
Saccharomyces pastorianus	CBS 1513		Z95950
Saccharomyces pastorianus	CBS 1538^T		Z95949
Williopsis saturnus var. *saturnus*	CBS 5761^T		Z93875
Williopsis saturnus var. *saturnus*	CBS 5761^T		Z93882
Williopsis saturnus var. *mrakii*	NCYC 500^T		Y11320
Williopsis saturnus var. *mrakii*	NCYC 500^T		Y11319
Williopsis saturnus var. *sargentensis*	CBS 6342^T		Z93879
Williopsis saturnus var. *sargentensis*	CBS 6342^T		Z93886
Williopsis saturnus var. *subsufficiens*	CBS 5763^T		Z93881
Williopsis saturnus var. *subsufficiens*	CBS 5763^T		Z93888
Zygosaccharomyces cidri	CBS 4575^T		Z48347
Zygosaccharomyces cidri	CBS 4575^T		Z48361
Zygosaccharomyces fermentati	CBS 707^T		Z48358
Zygosaccharomyces fermentati	CBS 707^T		Z48362

[a] ATCC, American Type Culture Collection, Manassas, Va.; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; IFO, Institute of Fermentation, Osaka, Japan; JCM, Japan Collection of Microorganisms, Saitama, Japan; M, Meiji Pharmaceutical University, Tokyo, Japan; MCO, Medical College of Ohio, Toledo, Ohio; NCYC, National Collection of Yeast Cultures, Norwich, United Kingdom; NIH, National Institutes of Health, Rockville, Md.

temperature, 53°C; flow rate, 1 ml/min; detection, 275 nm. Standard ubiquinones were used as references.

Serotyping. Serotyping was carried out only with the *Trichosporon* sp. strain M 9481 environmental isolate. The serotype of the isolate was determined by the cell slide agglutination test with specific factor sera as described by Ikeda et al. (9).

Nucleotide sequence accession numbers. The nucleotide sequences discussed in this paper have been deposited in the DNA Data Bank of Japan (DDBJ), and their accession numbers are given in Table 1.

RESULTS

Sequences of the ITS regions. Figures 1 and 2 show the nucleotide sequences of ITS 1, including the 3′ end of 18S rDNA, and ITS 2 of all the species in the genus *Trichosporon*. With the exceptions of *T. asahii* var. *coremiformis*, *T. asahii* var. *faecalis*, *T. brassicae*, *T. moniliiforme*, and *T. sporotrichoides*, these are consensus sequences built from the sequences of more than two strains of each species. ITS 1 was from 115 to 123 bp long, while ITS 2 was from 168 to 176 bp long. The within-species length variation in either ITS 1 or ITS 2 was not remarkable.

Sequence similarity between strains of a single species. Multiple strains of *T. asahii*, *T. mucoides*, *C. albicans*, and *S. cerevisiae* were examined to assess intraspecific variation (Table 2). In nine strains of *T. asahii*, <1- and 2-base differences were found in ITS 1 and 2, respectively, with overall similarities of 99.3 to 100% for both ITS 1 and 2. Five strains of *T. mucoides* had overall similarities of 98.9 to 100% in both the ITS 1 and 2 regions. For *C. albicans*, the similarities of the sequences were 99.7 to 100%. Although there were six different bases in the *S. cerevisiae* sequences in ITSs 1 and 2, the overall sequence similarity was 99.0%. Although strains in the same species do not necessarily have identical sequences, the overall sequence similarity of both ITSs 1 and 2 was 99% or more.

Relationship between the nuclear DNA relatedness value and ITS sequence similarity. Table 3 shows the matrices of sequence similarity for ITSs 1 and 2 for all of the species in the genus *Trichosporon*. The matrix for the overall ITS sequence is shown in Table 4. Fig. 3 shows the relationship between the nuclear DNA relatedness value and the sequences' ITS 1 and 2 similarities. This figure is based on the results for 74 pairs. A species concept has been defined on the basis of the nuclear DNA relatedness value, which corresponds well with biological relatedness (31). Within the same species (high-relatedness group), the value is approximately 80% or more. Species with values of approximately 40 to 80% are varieties of the same species or sibling species (intermediate-relatedness group). The value is less than 40% in different species (low-relatedness group). In the high-relatedness group, the sequence similarities of ITSs 1 and 2 were more than 98.9 and 98.8%, respectively. In the intermediate-relatedness group, the sequence similarities of ITSs 1 and 2 were more than 98.3 and 97.8%, respectively. In the low-relatedness group, data for ITS sequences with similarities of less than 90% were excluded from Figs. 3 and 4. The ITS similarities of the low-relatedness group were lower than the values for the high and intermediate groups. Exceptions were seen in *T. asahii* and *T. asteroides*. Their ITS 2 sequences were identical, but they had low nuclear DNA relatedness values of between 12 and 30% (37).

The relationship between the nuclear DNA relatedness value and the ITS sequences is shown in Fig. 4. The high-relatedness group had more than 99.0% similarity, while the intermediate-relatedness group had more than 97.9% similarity. In the intermediate group, 100% sequence similarities were found between *T. domesticum* and *T. montevideense* and between the two varieties of *C. famata*. The low-relatedness group showed less than 99.3% sequence similarity. No identical sequences were found in the low-relatedness group, but the similarities between *T. asteroides* and the three varieties of *T. asahii* and between *T. dulcitum* and *T. gracile* exceeded 99%.

Species-specific sequences. Table 5 shows the species-specific sequence used to distinguish each species. These sequences do not depend on the strain. The three varieties of *T.*

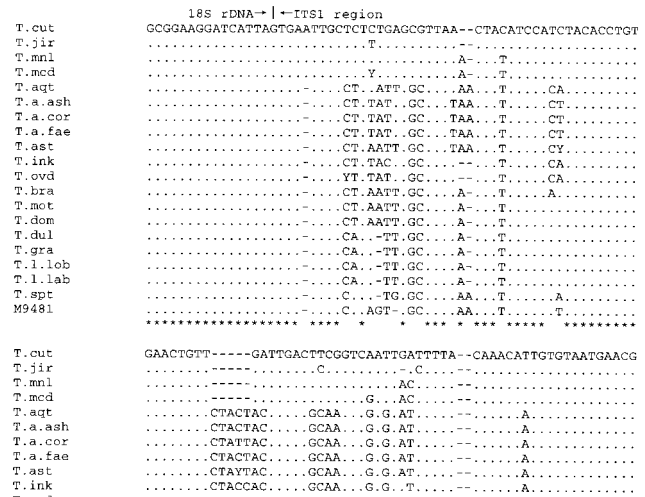

FIG. 1. Alignment of the *Trichosporon* ITS 1 sequences, including the 3′ end of 18S rDNA. Periods and asterisks are used when the nucleotide at a particular position is identical to that in *T. cutaneum*. Dashes represent deletions necessary for alignment. T. cut, *T. cutaneum*; T. jir, *T. jirovecii*; T. mnl, *T. moniliiforme*; T. mcd, *T. mucoides*; T. aqt, *T. aquatile*; T. a. ash, *T. asahii* var. *asahii*; T. a. cor, *T. asahii* var. *coremiformis*; T. a. fae, *T. asahii* var. *faecalis*; T. ast, *T. asteroides*; T. ink, *T. inkin*; T. ovd, *T. ovoides*; T. bra, *T. brassicae*; T. mot, *T. montevideense*; T. dom, *T. domesticum*; T. dul, *T. dulcitum*; T. gra, *T. gracile*; T. l. lob, *T. loubieri* var. *loubieri*; T. l. lab, *T. loubieri* var. *laibachii*; T. spt, *T. sporotrichoides*; M 9481, *Trichosporon* sp. strain M 9481.

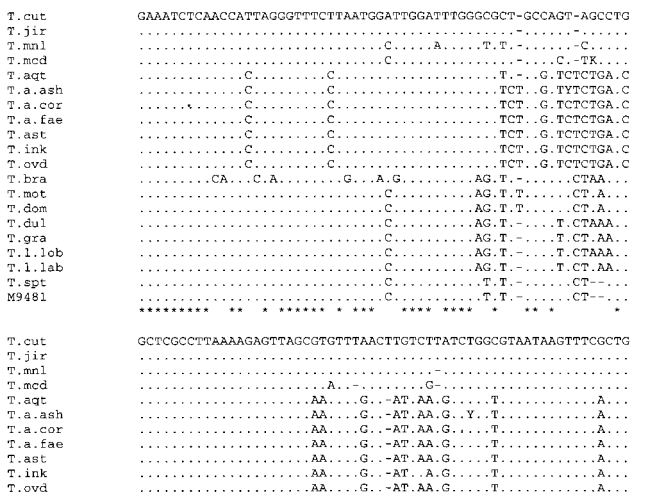

FIG. 2. Alignment of the *Trichosporon* ITS 2 sequences. For symbols and abbreviations, see the legend to Fig. 1.

asahii have identical sequences, so the specific sequences do not differentiate among them. *T. domesticum* and *T. montevideense* also have identical sequences and cannot be distinguished by ITS sequences.

Serotype and ubiquinone of *Trichosporon* sp. strain M 9481. The slide agglutination test with specific factor sera demonstrated that environmental isolate M 9481 was serotype II. This isolate had Q9 as the major ubiquinone.

Molecular phylogenetic analysis based on the ITS sequences. Figures 5 and 6 show the molecular phylogenetic trees based on the ITS 1 and 2 sequences constructed by the neighbor-joining and maximum-likelihood methods, respectively. The serotypes of the *Trichosporon* species are also shown in Fig. 5 and 6. The overall topology of the neighbor-joining tree is slightly different from that constructed by the maximum-likelihood method. This seems to be due to bootstrap values and differences in the method of analysis. However, the serotype correlated well with molecular phylogenetic trees, regardless of the ITS region or method. In the *T. sporotrichoides* clade, the strain M 9481 has serotype II, while *T. sporotrichoides* does not react with any factor sera.

DISCUSSION

To detect pathogenic fungi rapidly, oligonucleotide primers for PCR have been designed, based on the sequences of 18S or 26S rDNA (6, 46). These sequences have been determined for most pathogenic yeasts. The relatively high levels of sequence similarity observed among some species appears to limit the value of 18S or 26S rDNA for differentiating species that are phylogenetically closely related. The ITS region is located between the 18S and 26S rRNA genes and is subdivided into the ITS 1 region, which separates the 18S and 5.8S rRNA genes, and the ITS 2 region, which is found between the 5.8S and 26S rRNA genes. It is generally thought that the ITS regions have higher rates of divergence than the 18S, 5.8S, or 26S rRNA genes. The lengths of the 18S and 26S rRNA genes are essentially identical in all species, while the lengths of the ITS regions depend on the species. For example, the ITS 2 regions of *Candida glabrata* (U70498), *Candida kefyr* (U70502), and *S. cerevisiae* (Z75722) are approximately 230 to 240 bp long; those of *Candida guilliermondii* (U70499) and *C. famata* (U70500) are approximately 190 bp long; those of *C. albicans*

TABLE 2. Number of nucleotide differences in ITSs 1 and 2 within a single species

Species and strain	No. of differences			
	ITS 1	ITS 2	ITS 1 + 2	% similarity
Trichosporon asahii				
M 9306				
M 9309	1	0	1	100
M 9311	0	1	1	99.7
M 9312	0	0	0	100
M 9470	0	0	0	100
M 9474	0	1	1	99.7
M 9475	0	2	2	99.3
M 9476	0	0	0	100
M 9477	0	0	0	100
Trichosporon mucoides				
M 9422				
M 9331	2	1	3	98.9
M 9478	0	0	0	100
M 9479	0	0	0	100
M 9480	0	0	0	100
Candida albicans				
M 1001				
M 1016	0	1	1	99.7
M 1445	0	0	0	100
M 1447	0	0	0	100
M 1601	0	1	1	99.7
M 1602	0	1	1	99.7
M 2088	0	0	0	100
M 2089	0	0	0	100
M 2091	0	0	0	100
M 2093	0	0	0	100
Saccharomyces cerevisiae				
CBS 1171				
CBS 382	1	0	1	99.8
CBS 400	3	3	6	99.0
CBS 423	1	2	3	99.5
CBS 2247	1	0	1	99.8
CBS 3081	0	0	0	100
CBS 3093	0	0	0	100
CBS 4903	1	0	1	99.8
CBS 5378	0	0	0	100
CBS 5635	3	0	3	99.5

(L07796), *Candida tropicalis* (L11349), *Candida parapsilosis* (L11352), and *Candida viswanathii* (U70510) are approximately 130 to 140 bp long; and those of *Candida lusitaniae* (U70503) and *Candida rugosa* (U70506) are only 70 to 90 bp long (21). The clades in the molecular phylogenetic tree for these species correspond well to the ITS sequence length. ITSs 1 and 2 in the *Trichosporon* species are essentially the same size. With the exception of *T. pullulans*, the genus *Trichosporon* is monophyletic (35). *T. pullulans* was phylogenetically distinct from the other taxa in the genus, suggesting that this species does not belong in the genus. The lengths of the ITS 1 and 2 regions of *T. pullulans* are 40 and 50 bp longer, respectively, than those of the other *Trichosporon* species. As mentioned above, we believe that highly species-specific sequences can be found in the ITS sequences. To design highly specific oligonucleotide primers for PCR, sequence data for both the pathogenic and nonpathogenic species are required. Mannarelli and Kurtzman (23) developed a PCR-based identification system for the 14 *Candida* species that are human pathogens based on a ca. 600-nucleotide variable region (D1/D2) at the 5′-end of

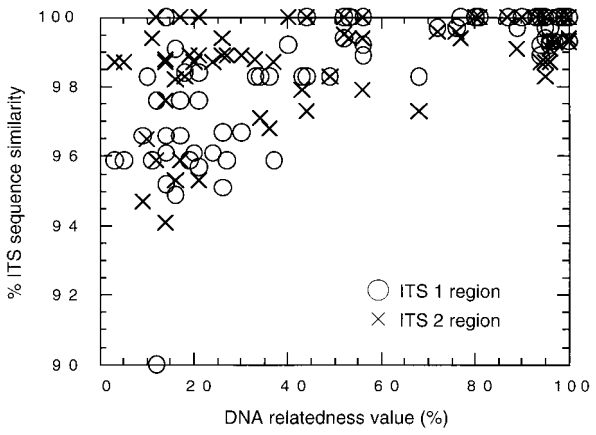

FIG. 3. Relationship between nuclear DNA relatedness value and similarities of ITS 1 and 2 sequences considered separately.

the 26S rDNA. Prior to this research work, their group determined the sequences of 204 *Candida* and related species, including nonpathogenic species (20). Their PCR system can clearly distinguish pathogenic species from species that are phylogenetically closely related.

DNA sequence can be determined quite rapidly with an automated DNA sequencer. Starting from DNA extraction from yeast cells, the ITS sequence can be determined within a working day, or 24 h at the most. Once an ITS sequence database has been constructed, rapid identification can be made. Since this identification method is not based on physiological characteristics, such as the carbon assimilation pattern, the chance of misidentification is reduced.

In this study, we found that conspecific species have less than a 1% overall nucleotide difference in both the ITS 1 and 2 regions. The species that have an intermediate DNA relatedness value have ITS sequences with 99% or more similarity, such as the three varieties of *T. asahii* and the two varieties of *C. famata*. Although *T. domesticum* and *T. montevideense* are distinct biological species, they have the same ITS sequences. They are considered to share an intermediate DNA relatedness value (42). With a few exceptions, the ITS sequence similarity between different species is less than 99.0%. The ITS

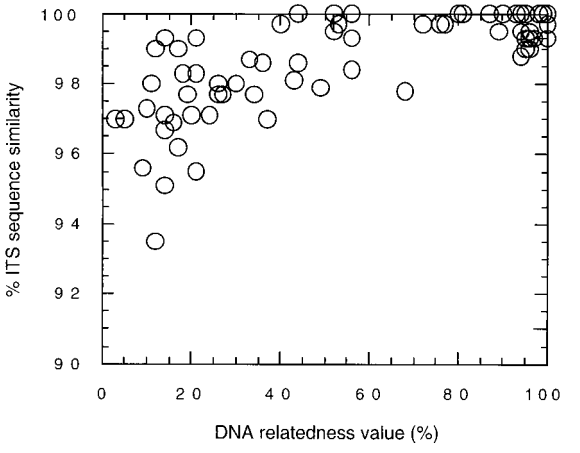

FIG. 4. Relationship between nuclear DNA relatedness value and similarity of combined ITS 1 and 2 sequences.

TABLE 3. Matrix of ITS 1 and 2 similarities for *Trichosporon* species[a]

Species	*T. cutaneum*	*T. jirovecii*	*T. moniliiforme*	*T. mucoides*	*T. asahii* var. *asahii*	*T. asahii* var. *coremiformis*	*T. asahii* var. *faecalis*	*T. aquatile*	*T. asteroides*	*T. ovoides*	*T. inkin*	*T. brassicae*	*T. montevideense*	*T. domesticum*	*T. dulcitum*	*T. gracile*	*T. loubieri* var. *laibachii*	*T. loubieri* var. *loubieri*	*T. sporotrichoides*	*Trichosporon* sp. strain M 9481
T. cutaneum		95.9	94.1	95.3																
T. jirovecii	96.6		94.7	98.2																
T. moniliiforme	96.6	95.7		92.9																
T. mucoides	95.7	94.9	99.1																	
T. asahii var. *asahii*						100	100	98.9	100	99.4	98.3									
T. asahii var. *coremiformis*					99.2		100	98.9	100	99.4	97.7									
T. asahii var. *faecalis*					100	99.2		98.9	100	99.4	98.3									
T. aquatile					96.7	95.9	96.7		98.9	98.9	97.7									
T. asteroides					97.6	98.4	97.6	97.6		99.4	98.9									
T. ovoides					95.9	95.1	95.9	97.6	94.3		98.3									
T. inkin					95.1	95.1	97.6	93.5	92.7	98.4										
T. brassicae																				
T. montevideense												92.5		100						
T. domesticum												92.5	100							
T. dulcitum																98.8	97.7	96.5		
T. gracile															100		98.8	97.1		
T. loubieri var. *laibachii*															98.3	98.3		98.3		
T. loubieri var. *loubieri*															98.3	98.3	96.6			
T. sporotrichoides																				98.2
Trichosporon sp. strain M 9481																			90.6	

[a] Similarities less than 90% are not indicated. Data in the upper right portion of the table refer to ITS 2 similarity, and data in the lower left portion refer to ITS 1 similarity.

TABLE 4. Matrix of overall ITS similarity for *Trichosporon* species[a]

Species	*T. cutaneum*	*T. jirovecii*	*T. moniliiforme*	*T. mucoides*	*T. asahii* var. *asahii*	*T. asahii* var. *coremiformis*	*T. asahii* var. *faecalis*	*T. aquatile*	*T. asteroides*	*T. ovoides*	*T. inkin*	*T. brassicae*	*T. montevideense*	*T. domesticum*	*T. dulcitum*	*T. gracile*	*T. loubieri* var. *laibachii*	*T. loubieri* var. *loubieri*	*T. sporotrichoides*	*Trichosporon* sp. strain M 9481
T. cutaneum																				
T. jirovecii	96.1																			
T. moniliiforme	95.1	95.1																		
T. mucoides	95.4	96.8	95.8																	
T. asahii var. *asahii*																				
T. asahii var. *coremiformis*					99.7															
T. asahii var. *faecalis*					100	99.7														
T. aquatile					98.0	97.6	98.0													
T. asteroides					99.0	99.3	98.0	98.3												
T. ovoides					98.0	97.6	98.0	98.3	97.3											
T. inkin					96.9	96.6	98.0	95.9	96.3	98.3										
T. brassicae																				
T. montevideense																				
T. domesticum													100							
T. dulcitum																				
T. gracile															99.0					
T. loubieri var. *laibachii*															97.9	98.6				
T. loubieri var. *loubieri*															97.2	97.6	97.6			
T. sporotrichoides																				
Trichosporon sp. strain M 9481																			95.1	

[a] Similarities of less than 90% are not indicated.

TABLE 5. Species-specific sequences in the ITS regions

Species	Species-specific sequence	Position of nucleotide
Medically relevant species		
T. asahii	TTTATAGGCTTAT	10–22[a]
T. asteroides	TTAATTGGCTTAT	10–22[a]
T. cutaneum	TCGGTCAATTGAT	60–72[a]
T. inkin	TTTACAGGCTTAA	10–22[a]
T. mucoides	TCGGTCGATTACT	61–73[a]
T. ovoides	TTTATAGGCTTAA	10–22[a]
Non-medically relevant species		
T. aquatile	CATTGGCTTAAAA	12–24[a]
T. brassicae	CGATTCAATTTTA	64–76[a]
T. domesticum (=*T. montevideense*)	CGGATTCGATTTT	64–76[a]
T. dulcitum	AAAGGAGTTAGCAAGTTTTACTAT	69–92[b]
T. gracile	AAAGGAGTTAGCAAGTTTAACTAT	69–92[b]
T. jirovecii	CCGGTCAATTACT	60–72[a]
T. moniliiforme	TCGGTCAATTACT	61–73[a]
T. loubieri var. *loubieri*	GATCATAACAAGA	103–115[a]
T. loubieri var. *laibachii*	GATCATAACTAA	102–103[a]
T. sporotrichoides	CCTCTGGGCTTAA	8–20[a]

[a] ITS 1.
[b] ITS 2.

sequence of *T. asahii* had two or three different bases from that of *T. asteroides* (99.0 to 99.3% similarity). It is very difficult to distinguish between these two species by using physiological characteristics (4), but the former is responsible for deep-seated infection while the latter is associated with superficial infection (4, 7).

Strain M 9481, which was isolated from a soil sample, was serotype II and had Q9 as the major ubiquinone. Although this

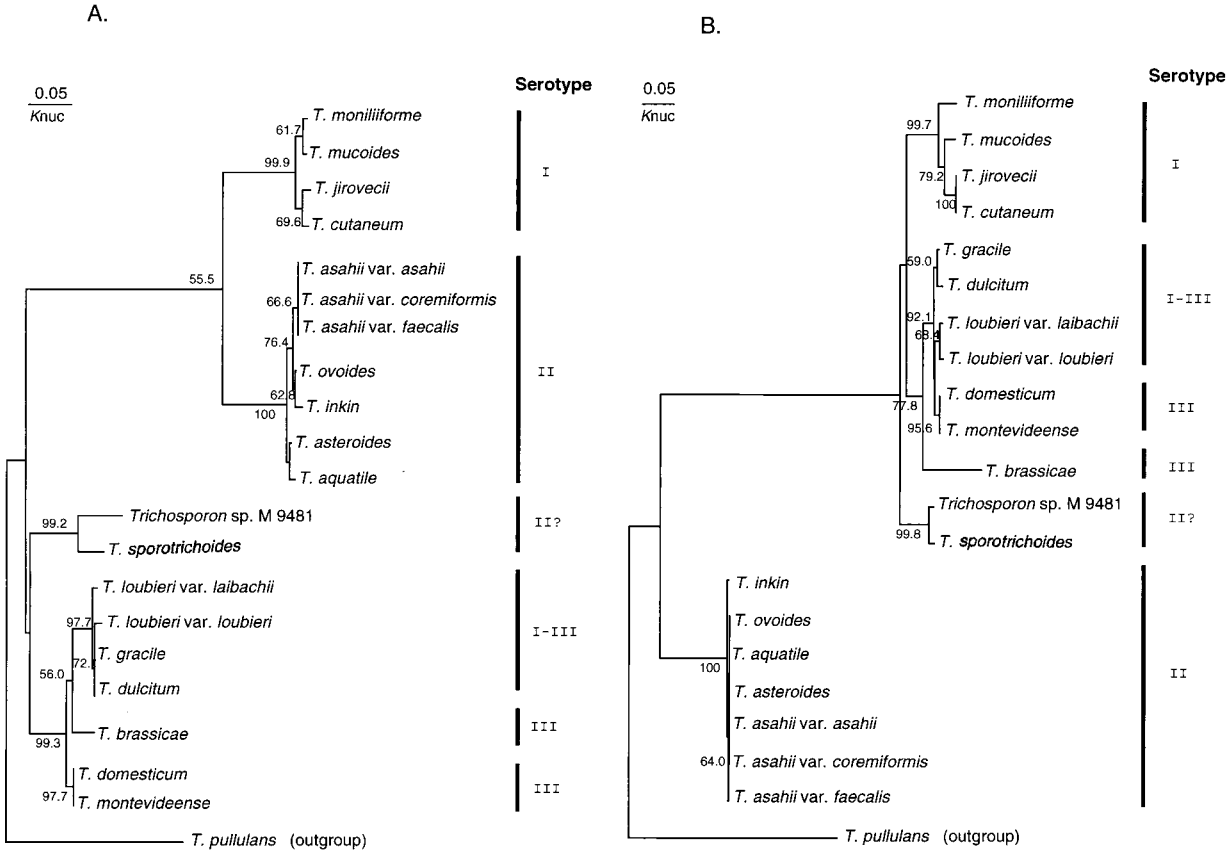

FIG. 5. Molecular phylogenetic trees based on the *Trichosporon* ITS 1 (A) and 2 (B) sequences. The trees were constructed by the neighbor-joining method. The numerals represent the confidence level from 1,000 replicate bootstrap samplings (frequencies less than 50% are not indicated). *K*nuc, Kimura's parameter (15).

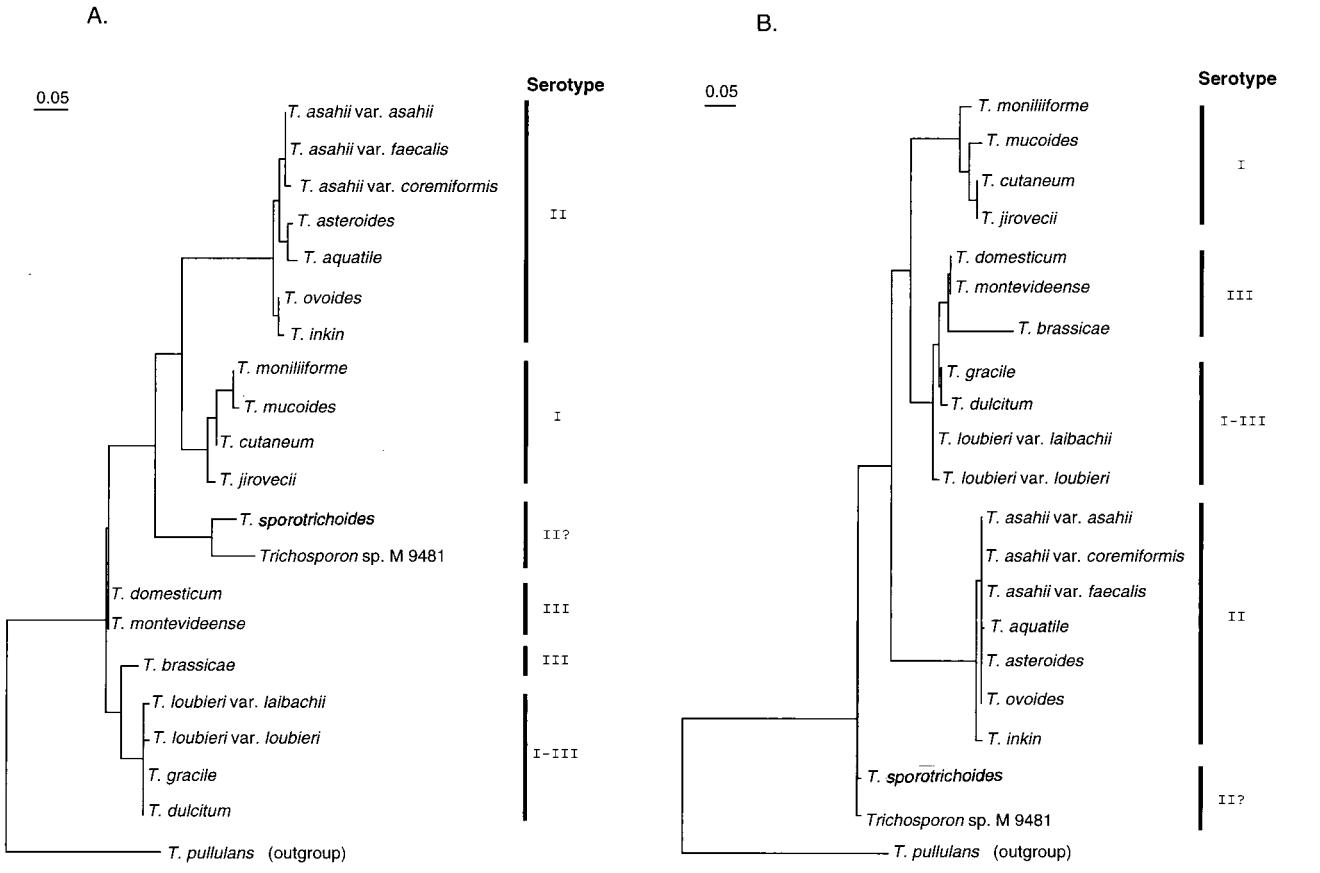

FIG. 6. Molecular phylogenetic trees based on the *Trichosporon* ITS 1 (A) and 2 (B) sequences. The trees were constructed by the maximum-likelihood method. The scale marker indicates the distance in relative units that equals 5% of the total branch length depicted.

strain is positioned in the *T. sporotrichoides* clade on the phylogenetic tree, its sequence similarity with the clade is only 95.1%. Strain M 9481 is considered a new species in the genus *Trichosporon*. At present, according to our data and a literature survey, the overall ITS sequence similarity of identical species is more than 99.0%. The accuracy of this value should be clarified as further data are accumulated. Kurtzman and Robnett (20) found a similar result in their analysis of the D1/D2 region of the 26S rDNA: the same species had fewer than 1% sequence differences.

The molecular phylogenetic trees of the genus *Trichosporon* constructed from the 18S and 26S rDNA sequences have already been reported (34, 35). The topology of these trees differs from that of the trees constructed from the ITS 1 and 2 sequences. However, the correlation between these trees depends on the molecular sequence and the molecular phylogenetic method. Although the factor sera we made are not species-specific, the serological groups correspond to the molecular phylogeny. Serological typing gives useful information to tentatively identify an isolate.

In conclusion, we constructed a readily used and accurate identification system for all of the species in the genus *Trichosporon*, including the six medically relevant species, based on comparative sequence analysis of the ITS regions. We expect that a sequence database will be constructed for other pathogenic fungi and related species.

ACKNOWLEDGMENTS

We thank Tomoe Ichikawa and Hiromi Shinohara for their technical skills in analyzing the ubiquinone.

REFERENCES

1. **Ando, M., K. Arima, R. Yoneda, and M. Tamura.** 1991. Japanese summer-type hypersensitivity pneumonitis: geographic distribution, home environment, and clinical characteristics of 621 cases. Am. Rev. Respir. Dis. **144:** 765–769.
2. **Ando, M., T. Sakata, and K. Yoshida.** 1990. Serotype-related antigen of *Trichosporon cutaneum* in the induction of summer-type hypersensitivity pneumonitis: correlation between serotype of inhalation challenge-positive antigen and that of the isolates from patients' homes. J. Allergy Clin. Immunol. **85:**36–44.
3. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
4. **Guého, E., L. Improvisi, G. S. de Hoog, and B. Dupont.** 1994. *Trichosporon* on humans: a practical account. Mycoses **37:**3–10.
5. **Guého, E., M. T. Smith, G. S. de Hoog, G. B. Grand, R. Christen, and W. H. Batenburg-van der Vegte.** 1992. Contributions to a revision of the genus *Trichosporon*. Antonie Leeuwenhoek **61:**289–316.
6. **Haynes, K. A., T. J. Westerneng, J. W. Fell, and W. Moens.** 1995. Rapid detection and identification of pathogenic fungi by polymerase chain reaction amplification of large subunit ribosomal DNA. J. Med. Vet. Mycol. **33:**319–325.
7. **Herbrecht, R., H. Koening, K. Waller, L. Liu, and E. Guého.** 1993. *Trichosporon* infections: clinical manifestations and treatment. J. Mycol. Med. **3:**129–136.
8. **Hoy, J., K. C. Hsu, K. Rolston, R. L. Hopfer, M. Luna, and G. P. Bodey.** 1986. *Trichosporon beigelii* infection: a review. Rev. Infect. Dis. **8:**959–967.
9. **Ikeda, R., M. Yokota, and T. Shinoda.** 1996. Serological characterization of *Trichosporon cutaneum* and related species. Microbiol. Immunol. **40:**813–819.

10. **Itoh, T., H. Hosokawa, U. Kohdera, N. Toyazaki, and Y. Asada.** 1996. Disseminated infection with *Trichosporon asahii*. Mycoses **39:**195–199.
11. **James, S. A., M. D. Collins, and I. N. Roberts.** 1996. Use of an rRNA internal transcribed spacer region to distinguish phylogenetically closely related species of the genera *Zygosaccharomyces* and *Torulaspora*. Int. J. Syst. Bacteriol. **46:**189–194.
12. **James, S. A., I. N. Roberts, and M. D. Collins.** 1998. Phylogenetic heterogeneity of the genus *Williopsis* as revealed by 18S rRNA gene sequences. Int. J. Syst. Bacteriol. **48:**591–596.
13. **Kamiyama, A., M. Niimi, M. Tokunaga, and H. Nakayama.** 1989. DNA homology between *Candida albicans* strains: evidence to justify the synonymous status of *C. stellatoidea*. Mycopathologia **107:**3–7.
14. **Kataoka-Nishimura, S., H. Akiyama, K. Saku, M. Kashiwa, S. Mori, S. Tanikawa, H. Sakamaki, and Y. Onozawa.** 1998. Invasive infection due to *Trichosporon cutaneum* in patients with haematologic malignancies. Cancer **82:**484–487.
15. **Kimura, M.** 1980. A simple method for estimation of evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16:**111–120.
16. **Kunova, A., J. Godal, J. Sufliarsky, S. Spanik, T. Kollar, and V. Krcmery, Jr.** 1996. Fatal *Trichosporon pullulans* breakthrough fungemia in cancer patients: report of three patients who failed on prophylaxis with itraconazole. Infection **24:**273–274.
17. **Kunova, A., D. Sorkovska, J. Sufliarsky, and V. Krcmery, Jr.** 1996. First report of catheter associated *Trichosporon pullulans* breakthrough fungemia in a cancer patient. J. Infect. **32:**70–71.
18. **Kurtzman, C. P.** 1990. DNA relatedness among species of the genus *Zygosaccharomyces*. Yeast **6:**213–219.
19. **Kurtzman, C. P.** 1991. DNA relatedness among saturn-spored yeasts assigned to the genera *Williopsis* and *Pichia*. Antonie Leeuwenhoek **60:**13–19.
20. **Kurtzman, C. P., and C. J. Robnett.** 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA gene. J. Clin. Microbiol. **35:**1216–1223.
21. **Lott, T. J., B. M. Burns, R. Zancope-Oliveira, C. M. Elie, and E. Reiss.** 1998. Sequence analysis of the internal transcribed spacer 2 (ITS2) from yeast species within the genus *Candida*. Curr. Microbiol. **36:**63–69.
22. **Makimura, K., Y. S. Murayama, and H. Yamaguchi.** 1994. Detection of a wide range of medically important fungal species by polymerase chain reaction (PCR). J. Med. Microbiol. **40:**358–364.
23. **Mannarelli, B. M., and C. P. Kurtzman.** 1998. Rapid identification of *Candida albicans* and other human pathogenic yeasts by using short oligonucleotides in a PCR. J. Clin. Microbiol. **36:**1634–1641.
24. **Martini, A. V.** 1989. *Saccharomyces paradoxus* comb. nov., a newly separated species of the *Saccharomyces* sensu stricto complex based upon nDNA/nDNA homologies. Syst. Appl. Microbiol. **12:**179–182.
25. **Martini, A. V., and C. P. Kurtzman.** 1985. Deoxyribonucleic acid relatedness among species of the genus *Saccharomyces* sensu stricto. Int. J. Syst. Bacteriol. **35:**508–511.
26. **Martini, A. V., and A. Martini.** 1987. Three newly delimited species of *Saccharomyces* sensu stricto. Antonie Leeuwenhoek **53:**77–84.
27. **Montrocher, R. M., C. Verner, J. Briolay, C. Gautier, and R. Marmeisse.** 1998. Phylogenetic analysis of the *Saccharomyces cerevisiae* group based on polymorphisms of rDNA spacer sequences. Int. J. Syst. Bacteriol. **48:**295–303.
28. **Nahass, G. T., S. P. Rosenberg, C. L. Leonardi, and N. S. Penneys.** 1993. Disseminated infection with *Trichosporon beigelii*. Arch. Dermatol. **129:**1020–1023.
29. **Nishikawa, A., H. Tomomatsu, T. Sugita, R. Ikeda, and T. Shinoda.** 1996. Taxonomic position of clinical isolates of *Candida famata*. J. Med. Vet. Mycol. **34:**411–419.
30. **Nishiura, Y., K. Nakagawa-Yoshida, M. Suga, T. Shinoda, E. Guého, and M. Ando.** 1997. Assignment and serotyping of *Trichosporon* species: the causative agents of summer-type hypersensitivity pneumonitis. J. Med. Vet. Mycol. **35:**45–52.
31. **Price, C. W., G. B. Fuson, and H. J. Phaff.** 1978. Genome comparison in yeast systematics: delimitation of species within the genera *Schwanniomyces*, *Saccharomyces*, *Debaryomyces*, and *Pichia*. Microbiol. Rev. **42:**161–193.
32. **Saito, N., and M. Nei.** 1987. Neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
33. **Shimazu, K., M. Ando, T. Sakata, K. Yoshida, and S. Araki.** 1984. Hypersensitivity pneumonitis induced by *Trichosporon cutaneum*. Am. Rev. Respir. Dis. **130:**407–411.
34. **Sugita, T., K. Makimura, A. Nishikawa, K. Uchida, H. Yamaguchi, and T. Shinoda.** 1997. Partial sequences of large subunit ribosomal DNA of a new yeast species, *Trichosporon domesticum* and related species. Microbiol. Immunol. **41:**571–573.
35. **Sugita, T., and T. Nakase.** 1998. Molecular phylogenetic study of the basidiomycetous anamorphic yeast genus *Trichosporon* and related taxa based on small subunit ribosomal DNA sequences. Mycoscience **39:**7–13.
36. **Sugita, T., A. Nishikawa, R. Ikeda, T. Shinoda, H. Sakashita, Y. Sakai, and Y. Yoshizawa.** 1998. First report of *Trichosporon ovoides* isolated from the home of a summer-type hypersensitivity pneumonitis patient. Microbiol. Immunol. **42:**475–478.
37. **Sugita, T., A. Nishikawa, and T. Shinoda.** 1994. Reclassification of *Trichosporon cutaneum* by DNA relatedness by using the spectrophotometric method and the chemiluminometric method. J. Gen. Appl. Microbiol. **40:**397–408.
38. **Sugita, T., A. Nishikawa, and T. Shinoda.** 1998. Rapid detection of species of the opportunistic yeast *Trichosporon* by PCR. J. Clin. Microbiol. **36:**1458–1460.
39. **Sugita, T., A. Nishikawa, and T. Shinoda.** 1998. Identification of *Trichosporon asahii* by PCR based on sequences of the internal transcribed spacer regions. J. Clin. Microbiol. **42:**475–478.
40. **Sugita, T., A. Nishikawa, T. Shinoda, and H. Kume.** 1995. Taxonomic position of deep-seated, mucosa-associated, and superficial isolates of *Trichosporon cutaneum* from trichosporonosis patients. J. Clin. Microbiol. **33:**1368–1370.
41. **Sugita, T., A. Nishikawa, T. Shinoda, and T. Kusunoki.** 1996. Taxonomic studies on clinical isolates from superficial trichosporonosis patients by DNA relatedness. Jpn. J. Med. Mycol. **37:**107–110.
42. **Sugita, T., A. Nishikawa, T. Shinoda, K. Yoshida, and M. Ando.** 1995. A new species, *Trichosporon domesticum*, isolated from the house of a summer-type hypersensitivity pneumonitis patient in Japan. J. Gen. Appl. Microbiol. **41:**429–436.
43. **Tashiro, T., H. Nagai, P. Kamberi, Y. Goto, H. Kikuchi, M. Nasu, and S. Akizuki.** 1994. Disseminated *Trichosporon beigelii* infection in patients with malignant diseases: immunohistochemical study and review. Eur. J. Clin. Microbiol. Infect. Dis. **13:**218–224.
44. **Tashiro, T., H. Nagai, T. Yamasaki, Y. Goto, S. Akizuki, and M. Nasu.** 1993. Disseminated *Trichosporon beigelii* infection: report of nine cases and review. Jpn. J. Infect. **67:**704–711.
45. **Thompson, J., J. D. Hompson, D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
46. **van Deventer, A. J. M., W. H. F. Goessens, A. van Belkum, H. J. van Vliet, E. W. M. van Etten, and H. A. Verbrugh.** 1995. Improved detection of *Candida albicans* by PCR in blood of neutropenic mice with systemic candidiasis. J. Clin. Microbiol. **33:**625–628.
47. **Yamada, Y., and K. Kondo.** 1973. Coenzyme Q system in the classification of the yeast genera *Rhodotorula* and *Cryptococcus*, and the yeast like genera *Sporobolomyces* and *Rhodosporidium*. J. Gen. Appl. Microbiol. **19:**59–77.
48. **Yamakami, Y., T. Tashiro, I. Tokimatsu, H. Nagai, H. Nagaoka, A. Hashimoto, Y. Goto, M. Nasu, T. Yamasaki, and M. Ito.** 1995. Microbiological and clinical study of fungemia between 1981 and 1992. Kansenshogaku Zasshi **69:**890–894.
49. **Yoshida, K., M. Ando, T. Sakata, and S. Araki.** 1988. Environmental mycological studies on the causative agent of summer-type hypersensitivity pneumonitis. J. Allergy Clin. Immunol. **81:**475–483.
50. **Yoss, B. S., R. L. Sautter, and H. J. Brenker.** 1997. *Trichosporon beigelii*, a new neonatal pathogen. Am. J. Perinatol. **14:**113–117.
51. **Walsh, T. J.** 1989. Trichosporonosis. Infect. Dis. Clin. N. Am. **3:**43–52.