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Sequence Analysis of the Ribosomal DNA Intergenic Spacer 1 Regions of *Trichosporon* Species

Takashi Sugita,[1]* Masamitsu Nakajima,[2]† Reiko Ikeda,[1] Toshiharu Matsushima,[2] and Takako Shinoda[1]

Department of Microbiology, Meiji Pharmaceutical University, Kiyose, Tokyo,[1] and Division of Respiratory Diseases, Department of Medicine, Kawasaki Medical School, Kurashiki, Okayama,[2] Japan

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We determined the sequence of the intergenic spacer (IGS) 1 region, which is located between the 26S and 5S rRNA genes, in 25 species of the genus *Trichosporon*. IGS 1 sequences varied in length from 195 to 719 bp. Comparative sequence analysis suggested that the divergence of IGS 1 sequences has been greater than that of the internal transcribed spacer regions. We also identified five genotypes of *T. asahii*, which is a major causative agent of deep-seated trichosporonosis, based on the IGS 1 sequences of 43 strains. Most of the isolates that originated in Japan were of genotype 1, whereas the American isolates were of genotype 3 or 5. Our results suggest that analysis of IGS regions provides a powerful method to distinguish between phylogenetically closely related species and that a geographic substructure may exist among *T. asahii* clinical isolates.

Fungal rRNA genes are tandemly repeated, with each repeat encoding 18S (small-subunit), 5.8S, and 26S (large-subunit) genes. Two other regions exist in each repeat: the internal transcribed spacer (ITS) region and the intergenic spacer (IGS) region (Fig. 1). Ribosomal DNA (rDNA) has been widely utilized for molecular systematics and the identification of microorganisms. The D1/D2 regions of 26S and ITS sequences have been used mainly to identify pathogenic fungi. At present, the 26S rDNA sequences of almost all yeasts, including nonpathogenic species, have been determined (3, 7, 8). The analysis of ITS sequences has been carried out mainly for pathogenic yeast species (1, 5, 9, 10, 16, 19). Peterson and Kurtzman (13) and Sugita et al. (16) demonstrated that a single species showed less than 1% dissimilarity in either the ITS region or D1/D2 26S rDNA. However, these sequence analyses are sometimes incapable of distinguishing between phylogenetically closely related species, such as the three varieties of *Cryptococcus neoformans*. Although three varieties within a single species can be distinguished for each varietal level by ITS sequence analysis, the distinction is based on differences of only three or four nucleotides (20). Recently, Diaz et al. (2) and Sugita et al. (17) demonstrated that three varieties of *C. neoformans* were more clearly distinguished by analysis of IGS 1 and IGS 2 sequences than by ITS sequence analysis. Therefore, IGS sequence analysis appears to be a powerful tool for differentiating between phylogenetically very closely related species.

The genus *Trichosporon* currently includes 25 species of basidiomycetous yeasts. Eight of these species are implicated in infectious or allergic diseases. *T. asahii*, *T. asteroides*, *T. cutaneum*, *T. inkin*, *T. mucoides*, and *T. ovoides* are involved in deep-seated or superficial infections (4, 6, 14), and *T. asahii*, *T. domesticum*, *T. montevideense*, and *T. mucoides* are associated with the allergic disease of summer-type hypersensitivity pneumonitis (12, 15). We have previously presented data on ITS sequences for the molecular identification of all members of the genus *Trichosporon*. However, this region is so highly homologous across the species that the genus *Trichosporon* may be considered phylogenetically monophyletic. Consequently, the differentiation of *Trichosporon* species requires the analysis of genes or regions that have greater divergence than the ITS. This paper describes the application of IGS sequence analysis to the identification of pathogenic species of *Trichosporon*.

The currently accepted 25 species of the genus *Trichosporon* were examined as shown in Table 1. *Trichosporon* DNA was extracted by the method of Makimura et al. (11). The IGS 1 region was amplified by PCR using the following oligonucleotide primers: 26SF, 5′-ATCCTTTGCAGACGACTTGA-3′, and 5SR, 5′-AGCTTGACTTCGCAGATCGG-3′. PCR was performed in a Thermocycler (model 9700; Applied Biosys-

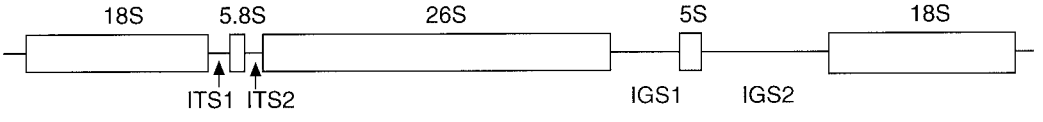

FIG. 1. Schematic representation of the rDNA locus in *Trichosporon*. Boxes indicate coding regions.

* Corresponding author. Mailing address: Department of Microbiology, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan. Phone: 81-424-95-8762. Fax: 81-424-95-8762. E-mail: sugita@my-pharm.ac.jp.

† Present address: Second Department of Internal Medicine, Hiroshima University School of Medicine, Hiroshima, Japan.

TABLE 1. Strains used and their IGS1 nucleotide sequence accession numbers

Species	Strain[a]	Source	Length of IGS 1 region (bp)	Accession no.
Trichosporon asahii genotype I	CBS 2479^T	Skin, Japan	485	AB066386
	M 9406	Urine, Japan	485	AB066392
	M 9415	Lung, Japan	485	AB066382
	M 9416	Lung, Japan	485	AB066381
	M 9417	Lung, Japan	485	AB066380
	M 9426	Feces, Japan	485	AB066376
	M 9432	Urine, Japan	485	AB066375
	M 9470	Blood, Japan	485	AB066384
	M 9474	Blood, Japan	485	AB066379
	M 9475	Intestinal fluids, Japan	485	AB066393
	M 9483	Blood, Japan	485	AB066389
	M 9485	Blood, Japan	485	AB066388
	M 9486	Blood, Japan	485	AB066390
	M 9496	Blood, Japan	485	AB066387
	M 9927	Urine, Japan	485	AB066377
	M 9928	Blood, Japan	485	AB066383
	M 9929	Urine, Japan	485	AB066391
	M 9930	Urine, Japan	485	AB066378
	M 9937	Blood, Japan	485	AB072602
	M 9938	Blood, Japan	485	AB072601
	M 9939	Blood, Japan	485	AB072599
	M 9940	Blood, Japan	485	AB072603
	M 9947	Blood, Japan	485	AB072604
	M 9948	Blood, Japan	485	AB072605
	M 9950	Blood, Japan	485	AB072600
	M 9951	Urine, Japan	485	AB066385
Trichosporon asahii genotype 2	M 9475	Blood, Japan	485	AB072606
Trichosporon asahii genotype 3	CBS 2530	*Mus musculus*, Brazil	490	AB066397
	CBS 4829	Feces, Brazil	490	AB071385
	M 9402	Blood, USA[b]	490	AB066396
	M 9403	Blood, USA	490	AB071383
	M 9941	Blood, USA	490	AB072607
	M 9942	Blood, USA	490	AB072608
	M 9943	Blood, USA	490	AB072609
	M 9944	Blood, USA	490	AB072610
	M 9945	Blood, USA	490	AB072611
Trichosporon asahii genotype 4	M 9474	Blood, Japan	485	AB066399
	M 9949	Blood, Japan	485	AB072612
Trichosporon asahii genotype 5	M 9410	Feces, USA	490	AB066401
	M 9411	Sputum, USA	490	AB071384
	M 9433	Urine, Japan	490	AB066402
	M 9935	Blood, USA	490	AB071386
	M 9936	Blood, USA	490	AB071387
Trichosporon aquatile	CBS 5973^T	Water	359	AB066403
	CBS 5988	Water	359	AB066404
Trichosporon asteroides	CBS 2481^T	Skin	466	AB066405
Trichosporon coremiiforme	CBS 2482^T	Lesion on head	478	AB066406
	M 9926	Soil	478	AB066409
	M 9932	Soil	478	AB066407
	M 9933	Soil	478	AB066408
	M 9934	Soil	478	AB066410
Trichosporon debeurumanianum	CBS 1896^T	Bronchial secretion	483	AB066411
Trichosporon dermatis	CBS 2043^T	Skin	357	AB066412
	M9946	House	357	AB072613
Trichosporon faecale	CBS 4828^T	Feces	490	AB066413
Trichosporon brassicae	CBS 6382^T	Cabbage	385	AB066414
Trichosporon cutaneum	CBS 2466^T	Skin lesion	331	AB066415
Trichosporon domesticum	M 9401^T	House	705	AB066416
	M 9421	House	705	AB066418
	M 9814	Cat	705	AB066417
Trichosporon dulcitum	CBS 8257^T	Soil	542	AB066419
	CBS 5785	Toadstool	542	AB066420
Trichosporon gracile	CBS 8189^T	Sour milk	523	AB066421
	CBS 8193	Teal	523	AB066422
Trichosporon guehoae	CBS 8521^T	Soil	268	AB066423
Trichosporon inkin	CBS 5585^T	Skin	489	AB066424
	CBS 7629	Urine	489	AB066425

Continued on following page

TABLE 1—*Continued*

Species	Strain[a]	Source	Length of IGS 1 region (bp)	Accession no.
Trichosporon japonicum	JCM 8357^T	Air	470	AB066426
Trichosporon jirovecii	CBS 6864^T	Toenail	328	AB066427
Trichosporon laibachii	CBS 5790^T	Soil		
	CBS 2495	Feces of *Rattus rattus*, authentic strain of *T. multisporum*		
Trichosporon loubieri	CBS 7065^T	Cow with mastitis	430	AB066428
Trichosporon moniliiforme	CBS 2467^T	Curdling milk	436	AB066429
	M 9813	Bird dropping	436	AB066430
Trichosporon montevideense	CBS 6721^T	Water purification tank	719	AB066431
	CBS 8261	Feces	719	AB066432
Trichosporon mucoides	CBS 7625^T	Meningitis patient	357	AB066433
Trichosporon ovoides	CBS 7556^T	Scalp	498	AB066434
Trichosporon porosum	CBS 2040^T	Exudate of yew tree	228	AB066435
	M 9481	Soil	228	AB066436
	M 9931	Soil	228	AB066437
Trichosporon sporotrichoides	CBS 8246^T	Soil	302	AB066438
Trichosporon veenhuisii	CBS 7136^T	Buffalo dung	401	AB066439
Trichosporon sp.	CBS 8645	Moist humus, around roots	195	AB066440

[a] CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; JCM, Japan Collection of Microorganisms, Saitama, Japan; M, Meiji Pharmaceutical University, Tokyo, Japan.
[b] USA, United States.

tems, Foster City, Calif.) with an initial 3-min denaturation at 94°C, followed by 30 cycles that consisted of 30 s at 94°C, 30 s at 57°C, and 1 min at 72°C, and a final 10-min extension at 72°C. The PCR products were sequenced with the 26SF and 5SR primers by using the ABI 310 DNA sequencer with an ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The lengths of the IGS 1 sequences of 24 *Trichosporon* species and their respective DDBJ accession numbers are listed in Table 1. The IGS 1 sequences ranged in length from 195 to 719 bp. For some unknown reason, the IGS 1 region of *T. loubieri* could not be amplified. Figure 2 shows a plot of the sequence similarities in the IGS and ITS regions for pairwise alignments between different species in the genus *Trichosporon*. The 99% similarity in ITS sequences observed between two species corresponds to approximately 55 to 95% IGS 1 sequence similarity. The 98% ITS sequence similarity in another pairwise comparison corresponds to approximately 45 to 55% IGS 1 sequence similarity. For example, *T. asahii* (GenBank accession no. AB018013), which is responsible for deep-seated infections, and *T. asteroides* (AB018017), which is associated with superficial infections, are 98.9% (295 out of 298 bp) similar in their ITS sequences. The similarity in the ITS region between *T. asahii* and the nonpathogenic species *T. coremiiforme* (AB018015) is 99.7% (297 out of 298 bp). However, within the IGS 1 region, *T. asahii* demonstrates 75.1 and 78.8% similarities to *T. asteroides* and *T. coremiiforme*, respectively. In addition, since the ITS sequences of *T. domesticum* and *T. montevideense*, which are the causative agents of summer-type hypersensitivity pneumonitis, are identical, these species could not be distinguished from one another by ITS sequence analysis (16). However, IGS sequence analysis of these two species reveals 94.6% sequence similarity. It is also noteworthy that the length of the ITS region, including the 5.8S region, ranges from 445 to 470 bp, while that of the IGS 1 region ranges from 195 to 704 bp. Since the members of the genus *Trichosporon* are phylogenetically very closely related, it appears that IGS sequence analysis is superior to ITS sequence analysis in differentiating *Trichosporon* species. The IGS sequence is divided into the IGS 1 and 2 regions. The complete IGS sequences of *C. neoformans* (L27078 and L27079) have been determined, and the IGS 1 and IGS 2 sequences of *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* are 68.1 and 84.2% similar, respectively. The *Malassezia* IGS 1 sequences are also more divergent than the IGS 2 sequences (unpublished data). We have not yet sequenced the IGS 2 of *Trichosporon* species, but preliminary results suggest that IGS 1 is more suitable than IGS 2 for the differentiation of phylogenetically closely related species.

Forty-three isolates of *T. asahii*, which is the major cause of deep-seated trichosporonosis, were obtained from various sources and geographic locations (Japan and the United

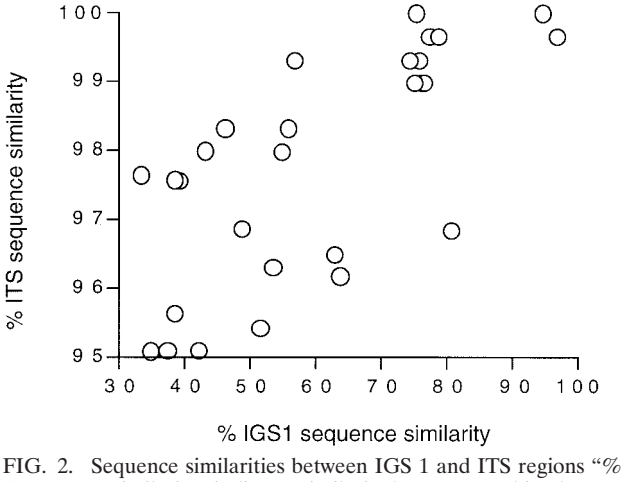

FIG. 2. Sequence similarities between IGS 1 and ITS regions "% ITS sequence similarity" indicates similarity between combined ITS 1 and ITS 2 sequences.

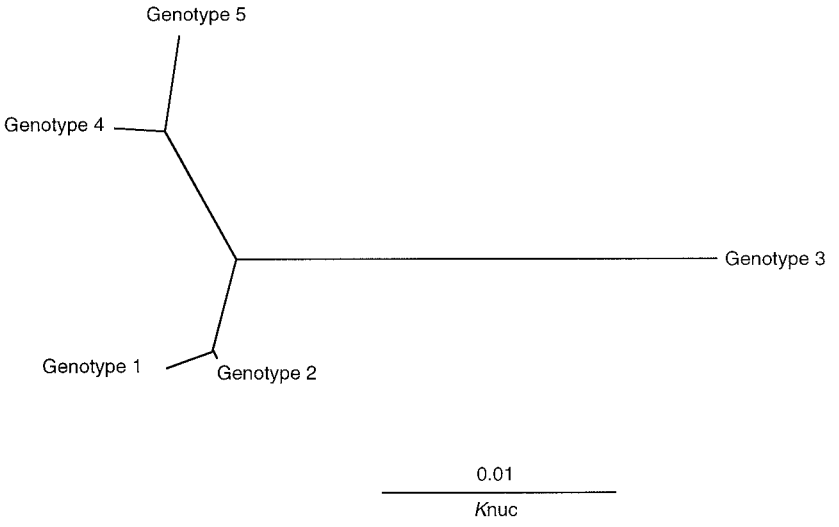

FIG. 3. A phylogenetic tree of five IGS 1 genotypes of *T. asahii*. The sequences were aligned using CLUSTAL W (version 1.8) software (18), and the tree was constructed using TreeView (version 1.6.2) (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

States) and analyzed (Table 1). The IGS 1 sequences ranged in length from 485 to 490 bp and were divided into five genotypes (Fig. 3). The genotypes shared between 95.1 and 98.8% similarity. Of the isolates that originated in Japan, 26 of 30 (87%) were genotype 1, while all 13 isolates from the United States were either genotype 3 or genotype 5. Genotypes 2 and 4 were found in only three isolates from Japan. No genotype 1 strains were found among the American isolates. Diaz et al. (2) found a geographic substructure among strains of *C. neoformans* var. *gattii*. Of the three genotypes, two corresponded to strains found in the United States, and the third represented Asian strains. We also found a correlation between the serotypes and genotypes of *C. neoformans* var. *gattii* strains in an analysis of both the IGS 1 and IGS 2 regions (17). Of the three genotypes, two consisted solely of serotype B strains, while the third consisted of both serotype B and serotype C strains. Although our study dealt with a limited number of strains, the IGS sequence analysis suggests that there is a correlation between the genotype and the geographical substructure of the *T. asahii* clinical isolates. Unfortunately, we could not obtain *T. asahii* clinical isolates from European countries. A comparison of the genotypes of strains from Europe should prove interesting.

We examined the IGS sequences of all members of the genus *Trichosporon* and concluded that IGS sequence analysis was superior to ITS sequence analysis in differentiating phylogenetically closely related species. IGS sequence analysis also shows great potential as a new epidemiological tool.

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