Fungal Identification Method Based on DNA Sequence Analysis: Reassessment of the Methods of the Pharmaceutical Society of Japan and the Japanese Pharmacopoeia

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Fungal identification methods based on DNA sequence analysis were recently published in *Methods of Analysis in the Health Sciences* (*Eisei-shiken-ho* in Japanese) issued by the Pharmaceutical Society of Japan (PSJ) and in the draft second supplement to the 14th edition of the *Japanese Pharmacopoeia* (JP). We carefully compared and assessed the effectiveness of the two methods of fungal identification. The PSJ method analyzes D1/D2 26S rDNA, while the JP method analyzes the internal transcribed spacer (ITS) 1 region. The former method is superior to the latter in that the identification criterion described in the PSJ method is rationally defined based on the species concept, whereas distinct species can be misidentified as being of the same species using the identification criterion described in the JP method; and there are more sequence data for the D1/D2 26S rDNA regions in the DNA data libraries (DDBJ/GenBank/EMBL) than for ITS1 data. Based on our assessment, we conclude that the PSJ method is superior to the JP method for fungal identification.

Key words —— fungi, identification, DNA sequence

INTRODUCTION

There are approximately 70000 to 80000 species of fungi. While filamentous fungi are identified using mainly morphological characteristics, yeasts are identified using biochemical characteristics, such as their ability to utilize carbon and nitrogen compounds. However, these methods of identification are often problematic as there can be different morpho/biotypes within a single species. They are also time-consuming, and require a great deal of skill. To resolve these problems, the Health Pharmaceutical Committee of the Pharmaceutical Society of Japan (PSJ) proposed a "Fungal identification method based on DNA sequence analysis," which was subsequently described in the *Methods of Analysis in the Health Sciences* (*Eisei-shiken-ho* in Japanese) issued by the PSJ in 2002.[1] In this method, a partial region of the 26S subunit of the fungal rRNA gene is sequenced and compared with known fungal DNA sequences. As DNA sequence analysis methods are objective, reproducible, and rapid means of identification, they have been widely used.[2–4] Recently, the "Rapid identification of microorganisms based on a molecular biological method" was proposed in the draft of the second supplement to the 14th edition of the *Japanese Pharmacopoeia* (JP).[5,6] However, there are marked differences between the PSJ and JP methods (Table 1). In this study, we compared and assessed the effectiveness of the two methods of fungal identification.

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MATERIALS AND METHODS

Nucleotide Sequence Similarity —— Sequence similarities were compared using nuclear DNA relatedness values taken from the literature for the internal transcribed spacer (ITS) 1 and D1/D2 26S rDNA sequences separately.[7] Sequence similarity was determined visually from pairwise alignments. The nucleotide sequences used in this study were obtained from the DNA Data Bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp).

Accumulation of DNA Sequences in DNA Data Libraries —— All 685 species listed in the 4th edition of The Yeasts, A Taxonomic Study[8] were

Table 1. Major Differences between the PSJ and JP Methods

Item	PSJ method	JP method
Gene or region analyzed	Domains 1 and 2 of the 26S subunit (D1/D2 26S rDNA) (see Fig. 1)	Internal transcribed spacer 1 region (ITS1) (see Fig. 1)
Identification criterion	Conspecific strains have more than 99% nucleotide similarity in the D1/D2 26S rDNA sequence	Conspecific strains have more than 90% nucleotide similarity in the ITS1 region

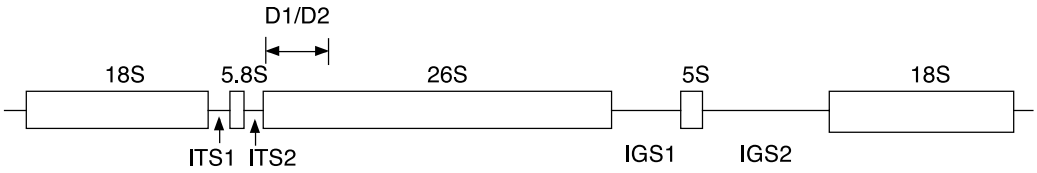

Fig. 1. Schematic Representation of the Fungal rRNA Gene
ITS, internal transcribed spacer; IGS, intergenic spacer; D1/D2, domains 1 and 2.

used in this study. The numbers of ITS1 and D1/D2 26S rDNA data that have been released from the DDBJ were examined. 18S rDNA sequences were also examined as a reference.

RESULTS AND DISCUSSION

The fungal rRNA gene consists of the 18S, ITS1, 5.8S, ITS2, 26S, intergenic spacer (IGS) 1, 5S, and IGS2 regions (Fig. 1). The major differences between the PSJ and JP methods are summarized in Table 1.

Identification Criteria

There are approximately 70000 to 80000 species of fungi. Of these, the sequences of 5.8S, 18S, D1/D2 26S rDNA, ITS, and IGS regions of almost all *Trichosporon* species have already been deposited in the DDBJ. As *Trichosporon* species are responsible for opportunistic infections, and are used as microbial biochemical oxygen demand (BOD) sensors, they are important yeasts from both the clinical and hygienic perspectives. In yeast taxonomy, a species concept has been defined using nuclear DNA relatedness values determined by a DNA-DNA hybridization experiment, which correspond well to biological relatedness.[9] Within a species, the DNA relatedness value is 70% or more (high relatedness group). Taxa with values of 40% to 70% are varieties of the same species or are sibling species (intermediate relatedness group). Between different species, the relatedness value is less than 40% (low relatedness group). Figure 2 shows the relationships between the nuclear DNA relatedness value and ITS1 and D1/D2 26S rDNA sequence similarities for members of the genus *Trichosporon*. Figure 2 is based on the results for 34 pairs. In the high-relatedness group, the ITS1 and D1/D2 26S rDNA sequence similarities exceed 99%. The JP method defines conspecific strains as having more than 90% similarity in the sequence of the ITS1 region, but there are distinct species that show more than 95% sequence similarity (Fig. 2). This suggests that comparing the DNA sequence similarity in the ITS1 region will lead to microorganisms in distinct species being misidentified as belonging to the same species. The identification criterion described in the JP method does not appear to be based on rational evidence, especially as regards the species concept. As far as we know, there have been no reports supporting the JP criterion.

In addition, analysis of D1/D2 26S rDNA sequences has the advantage that it not only enables species identification, but also permits phylogenetic analysis, unlike analysis of ITS1. Even when DNA sequence data from fungal isolates have not been registered in DNA data libraries and the isolate cannot be identified to the species level, the genus or family of the isolate can be identified from molecular phylogenetic analyses using D1/D2 26S rDNA sequences. In contrast, it is very difficult to deduce the phylogenetic position of a genus or family of an isolate from the ITS regions as they are more diverse than the 26S region.

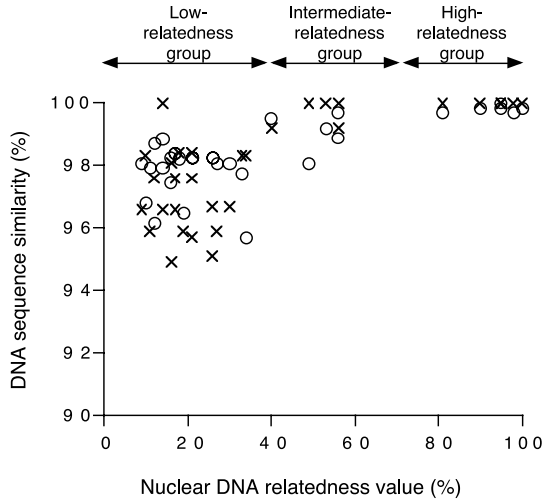

Fig. 2. Relationships between the Nuclear DNA Relatedness Value and the Similarities of the ITS1 and D1/D2 26S rDNA sequences
×, ITS1; ○, D1/D2 26S rDNA.

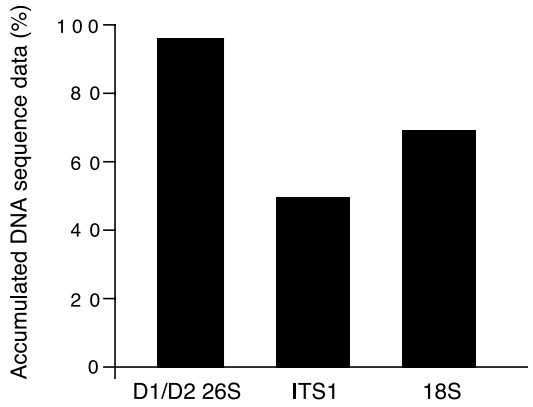

Fig. 3. Accumulated DNA Sequence Data in the DDBJ

Accumulation of DNA Sequences in the DDBJ

As there are an enormous number of filamentous fungi and their taxonomy is not well established, we focused on the sequence similarity in yeast species. Figure 3 shows accumulated DNA sequence data for ITS1, D1/D2 26S rDNA, and 18S rDNA in the DDBJ as of July 3, 2003. D1/D2 26S rDNA data of 666 species in a total of 685 yeast species identified have been released, while ITS1 data are available for only half of the species. Using 18S rDNA sequences, a limited number of fungi species can be identified. However, 18S rDNA sequence analysis has not been widely used for fungal identification because approximately 1800 bp must be determined for conclusive results. Nevertheless, 18S rDNA data have been released for 70% of species. Since the D1/D2 26S rDNA sequences of most yeast species have already been determined, the identification of the fungi using these sequences is practical.

In conclusion, the PSJ method is superior to the JP method as a standard fungal identification method.

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