Mycobiology 33(2): 109-112 (2005)
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Sequence Analysis of the Internal Transcribed Spacer of Ribosomal DNA in the Genus *Rhizopus*

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(Received April 13, 2005)

The internal transcribed spacer (ITS) regions including the 3'-end of 18S rRNA gene, 5.8S rRNA gene and the 5'-end of the 28S rRNA gene of *Rhizopus* spp. were amplified by PCR and analyzed by DNASIS program. Length polymorphism of these region ranged from 564 bp in *R. oryzae* to 789bp in *R. stolonifer*. The length and sequence of 5.8S was very conserved with 154~155 bp. The sequence of ITS2 was more variable than that of ITS1. The base substitution rates were ranged from 0 to 0.6069 per site, and higher rate was found in *R. stolonifer*. In general, transition was usually more frequent than transversion. On the basis of sequencing results, four groups were clustered with value of 61.9% similarity; *R. oryzae*, *R. microspores*, *R. homothallicus*, and *R. stolonifer* groups.

KEYWORDS: Base substitution, Internal transcribed spacer (ITS), *Rhizopus*, Sequence analysis

Rhizopus is a small genus of Zygomycetes (Mucorales). *Rhizopus* spp. produce a variety of enzymes, proteins, and by-products. About 82 strains have been investigated with very divergent views on the recognition of most taxa by different authors (Shipper, 1984). There are very few molecular techniques currently in use for the *Rhizopus*, and there is still very little experimental data available.

Molecular techniques are being employed more extensively for determining taxonomic assignments. The use of DNA complementarity studies (Ellis, 1985) and PCR or other molecular techniques are providing the basis for the assignment of fungi into their appropriate class, family, genus, species, and variety (Sutton, 1998).

ITS sequence comparisons are becoming an increasingly popular tool for phylogenetic analysis and for the differentiation of populations. DNA sequencing is generally accepted to be the more reliable method for revealing genetic relationship and could be used to evaluate relationships of organisms at any taxonomic rank. The internal transcribed spacer of rDNA is considered to be a variable region among genera and even among species (Paul, 2002). The degree of variability can be used in the classification of species or varieties (Su *et al.*, 1999). As a preliminary report of this work, PCR-RFLP analysis of twenty strains in *Rhizopus* spp. showed the polymorphism in length and restriction site of ITS (Park *et al.*, 2003) and a total of five ITS haplotypes were identified among these strains. To compare the result obtained from RFLP loci for the same strains, one or two strains representing the each haplotype were sequencing. For the purpose of clarifying the taxonomic status and genetic relationships which has been issued on the morphological classification, we compared the levels of DNA sequence divergence among the ITS regions of *Rhizopus* spp.

Materials and Methods

Fungal strains and culture condition. The information for strains of the *Rhizopus* spp. used this study is given at Table 1. All species were maintained at 4°C on potato dextrose agar (PDA). Mycelium for DNA extraction was grown in 250 *ml* of PDB (potato dextrose broth) in a rotary shaker on 180 rpm for 24 h at 28°C. After vacuum filtration, the mycelia were lyophilized, ground with sea sand and stored at −20°C. Genomic DNA for PCR was extracted according to the method of Lee *et al.* (2000).

Sequencing of entire ITS region. For sequencing, ITS region of the rDNA was amplified using primer ITS1 (5'-TCCGTTGGTGAACCAGCGG-3') and primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). Amplification was performed in 100 μl of reaction mixture containing 10 μl of 10 × buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1% Triton X-100], 2.5 units Ex *Taq* DNA polymerase (Takara, Japan), 200 μM dNTPs, 2 mM $MgCl_2$, and 10 pmol of both primers, ITS 1 and ITS4. The mixture was subjected to PCR in an MWG-Biotech (Germany). Using a TOPO TA Cloning kit (Invitrogen, USA), the amplified PCR products and the pCR2.1 vector were ligated and the resulting plasmids were introduced into *Escherichia coli*. Transformed cells were chosen at random from an LB agar plate (to which 50 μg

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Table 1. List of *Rhizopus* spp. examined in this study

Strains	Isolate number	Source[a]	Synonym	haplotypes[b]
R. oryzae Went et prinses-Geerligs	11145	ATCC	*R. arrhizus*	I
R. oryzae Went et prinses-Geerligs	22580	ATCC	*R. javanicus*	I
R. oryzae Went et prinses-Geerligs	24794	ATCC	*R. japonicus*	II
R. sexualis. sexualis (Smith) Callen	42542	ATCC		II
R. homothallicus Hesseltine et Ellis	42221	ATCC		III
R. oryzae Went et prinses-Geerligs	4772	IFO	*R. chinensis*	IV
R. microsporus var. *oligosporus*	22959	ATCC	*R. oligosporus*	IV
R. stolonifer (Ehrenberg: Fries) Lind	6227b	ATCC	*R. nigricans*	V

[a]ATCC : American Type Culture Collection, IFO : Institute for Fermentation, Osaka, Japan.
[b]Park *et al.*, 2003.

Table 2. The size (bp) and G+C content (%) of ITS 1, 5.8S and ITS2, respectively. The 3'-end of the 18S gene and 5'-end of the 28S gene was not calculated

Isolates	ITS1		5.8S		ITS2		Total size	Accession number
	size	G+C	size	G+C	size	G+C		
R. oryzae 22580	199	39.69	155	39.99	210	41.25	564	AF 543520
R. oryzae 11145	199	39.69	155	39.99	210	41.25	564	AF 543519
R. oryzae 24794	199	39.69	155	39.99	210	41.25	564	AF 543522
R. sexualis 42542	199	39.69	155	39.99	210	41.25	564	AF 543521
R. homothallicus	227	42.72	154	42.19	190	38.42	571	AF 543525
R. oryzae 4772	251	41.26	155	40.25	222	32.87	628	AF 543524
R. micro var. *oligosporus* 22959	251	41.26	155	40.25	222	32.87	628	AF 543523
R. stolonifer 6227b	292	32.87	154	40.89	343	23.61	789	AF 543526

ampicillin had been added), and extracted plasmid by Qiagen mini-prep columns. For sequencing [ABI PRISM® 3100 Genetic Analyzer (Applied Biosystem)], M13 forward and M13 reverse primers were used to amplify the template, and sequencing was carried out using plasmid-specific and insert-specific primers.

Sequences analysis. Sequences analyses were carried out using programmes within DNASIS (version 2.1). Phylogenetic tree was obtained from multiple align plot of ITS sequences among *Rhizopus* species studied. The sequence identity (%) between species was showed on the tree and the G+C content (%) were calculated. Pair-wise comparisons were calculated from the data matrix using maximum-matching method to generate a similarity matrix among strains. Kimura's two-parameter method was used to calculate the total number of nucleotide substitutions per site (K): $K = -0.5 \ln [(1-2P-Q)\sqrt{1-2Q}]$, where P is the proportion of transitions, and Q is the proportion of transversion (Kimura, 1980). The sequences for all species examined have been deposited in GenBank and taken accession numbers.

Results and Discussion

The ITS sequence of the different strains were PCR amplified using primers with ITS1 and ITS4 primer. The PCR fragments were cloned, and several indepent clones of each strains were sequenced. These revealed a length polymorphism at the sequence level ranged from 564 to 789 bp.

The sizes of th ITS1 and ITS2 regions varied among strains from 199 bp to 292 bp and 190 bp to 343 bp, respectively (Table 2). The length of ITS regions including 5.8S for *R. stolonifer* was found to be considerably longer (789 bp) than those of other strains.

The total G+C contents of ITS1 and ITS2 varied between 32.87 to 42.72% in the ITS1 and 23.61 to 41.25% in the ITS2. The spaces of *R. stolonifer* had a very low G+C content with 32.87% in ITS and 23.61% in ITS2. When the non-coding regions of Zygomycete *Mucor miehei* were analyzed (Maicas *et al.*, 2000), ITS (198 bp) and ITS2 (255 bp) are A-T rich (66% and 77%, respectively) and ITS1 is longer than the corresponding sequence of other related zygomycota (Maicas *et al.*, 2000). But, in this study, ITS1 and ITS2 of *M. miehei* is still shorter than those of *R. stolonifer*. The length and G+C content of 5.8S in *M. miehei* and genus *Rhizopus* were very similar; 158 bp long, 40.5 G+C for *M. miehei* and 154~155 bp long, 39.99~40.89 G+C for the genus *Rhizopus*. In contrast, G+C contents of ITS region in *Fusarium solani* showed relatively high G+C contents; 46.70~51.30% in ITS1 region and 52.01~68.96% in ITS2 (Lee *et al.*, 2000). Takamatsu *et al.* (1998) reported that the secondary structure shown on the ITS region of powdery mildew fungi was supported by high G+C contents. It has been reported

Table 3. Matrices for the average numbers of substitutions per site

	1	2	3	4	5	6	7	8
1								
2	0							
3	0.2908	0.2908						
4	0.2908	0.2908	0					
5	0.2934	0.2934	0.0017	0.0017				
6	0.2934	0.2934	0.0017	0.0017	0			
7	0.2481	0.2481	0.2560	0.2560	0.2584	0.2584		
8	0.5733	0.5733	0.5117	0.5117	0.5153	0.5153	0.6069	

Number: 1. *R. microsporus* var. *oligosporus* 22959, 2. *R. oryzae* 4772, 3. *R. oryzae* 22580, 4. *R. oryzae* 11145, 5. *R. sexualis* 42542, 6. *R. oryzae* 24794, 7. *R. homothallicus* 42221, 8. *R. stolonifer* 6227b.

that ITS region form secondary structures, which are actively involved in the processing of the primary transcript in yeast (Rau and Planta., 1995). ITS1 is required for the processing of the 3' end of the 18S and the 5' end of the 5.8S molecules, while ITS2 is required for the processing of the 3' end of the 5.8S and the 5' end of the 28S molecules. The yeast results furthermore that the secondary-structure of these spaces is important for the processing reaction (van der Sande *et al.*, 1992). We could detect the restriction sites of restriction enzymes and coincided with ITS-RFLP analysis (Park *et al.* 2003). We have identified some more highly conserved regions in ITS1 than ITS2 sequence. The 3'-termini of the 18S rRNA sequence from 8 strains showed complete homology. No information about ITS region involving 5.8S sequence from any other *Rhizopus* species was found in the databases.

The nucleotide sequence data have been lodged in the EMBL nucleotide sequence database under the accession numbers shown at Table 2.

The range of sequence variation was from single and multi base pair change to multiple changes including deletions and insertions. Most variation of sequences in the 5.8S rRNA gene, was shown in *R. stolonifer* and *R. homothallicus.* The single base pair changes in the position 346 (T-C), 431 (G-A), 448 and 452 (T-C), 448, 453 (A-G), 464 (C-T) in *R. stolonifer* and in the position 405 (deletion), 448 (T-C), 452 (T-C), 473 (T-C) in *R. homothallicus* were observed. In general, transition was usually more frequent than transversion.

We have used the alignment to calculate the average number of nucleotide substitutions per site for the whole spacer sequences. This was done in pair wise sequence comparisons by using the method of Kimura (1980). The ITS is useful for examining the rates of base substitution among closly related taxa and for studying fixation by molecular drive. The average number of substitutions is apparently correlated with the evolutionary distance of the species within the *Drosophila melanogaster* species group. In *Drosophila*, average base substitution number per site for seven species was ranging from 0.014 to 0.433 (Scholtterer *et al.*, 1994). In *Rhizopus*, the base substitution number per site was ranged from 0 to 0.6069 (Table 3). Substitution number between *R. microsporous* and *R. oryzae* 4772 and those of between *R. sexualis* 42542 and *R. oryzae* 24794 showed zero. The highst was found in *R. stolonifer* which is biggest species in the genus *Rhizopus.* Unfortunately, we could not compare with related data about other fungi.

Table 4. Nucleotide sequence identities among each strains using Maximum matching program

Taxon	2	3	4	5	6	7	8
1	1.00	0.99	0.99	0.74	0.69	0.69	0.57
2		0.99	0.99	0.74	0.69	0.69	0.57
3			1.00	0.74	0.69	0.69	0.58
4				0.74	0.69	0.69	0.58
5					0.76	0.76	0.57
6						1.00	0.58
7							0.61

Number : 1. *R. oryzae* 22580, 2. *R. oryzae* 11145, 3. *R. oryzae* 24794, 4. *R. sexualis* 42542, 5. *R. homothallicus*, 6. *R. oryzae* 4772, 7. *R. microsporus* var. *oligosporus* 22959, 8. *R. stolonifer* 6227b.

For getting the similarity among these strains, the maximum-matching method on the DNASIS program was used (Table 4). The Similarity of 5.8S rDNA was 95~100%, whereas those of ITS1 and ITS2 were 50~99% and 39~100% respectively. It was proved that the 5.8S rRNA gene was highly conserved among strains, whereas ITS region was variable.

The phylogenetic tree obtained from multi-alignment plot was shown in Fig. 1. The cluster analysis separated the 8 strains into four major groups based on above 61.9% similarity; *R. oryzae*, *R. microspores*, *R. homothallicus* and *R. stolonifer* groups. The results of cluster analysis were coincided with those of PCR-RFLP analysis. The strains belonging to haplotypes I and II showed 99.8% similarity. *R. stolonifer* 42221 showed lowest similarity 47.9%, with other strains on tree. The similarity between *R. micro* var. *oligo* and *R. homothallicus* was 61.9%.

When taxonomical study of the genus *Rhizopus* was made by examining the morphological, cultural and physiological characteristics of 449 culture strains, Inui *et al.*

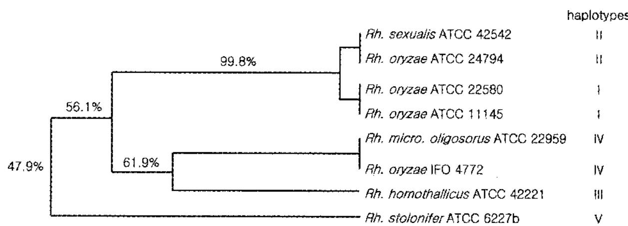

Fig. 1. The phylogenetic tree based on the nucleotide sequence multialignment of the ITS region of the *Rhizopus* spp. Numbers on the tree nodes indicate percentage of the similarity.

(1965) divided the genus *Rhizopus* into 4 section, 13 species and one new variety; section *Sexualis*, section *Nigricans* (referred to stolonifer), section *Oryzae* and section *Chinensis*.

Schipper (1984) did not accept the taxonomic decisions in the monograph of the genus by Inui *et al.* (1965). Instead, Schipper divided the genus *Rhizopus* into *R. stolonifer*, *R. oryzae* and *R. microspous* groups. According to Schipper (1984), the homothallic species *R. sexualis* belonging to *R. stolonifer* group resembles *R. stolonifer* in the shape and size of its sporangiospores, but differs in having relatively small sporangia and small sporangiophores. In our study, *R. sexuals* ATCC 42542 was distantly related to *R. stolonfer* and showed above 99.8% similarity with *R. oryzae. R. microsporus* revised by Schipper and Stalpers (1984) was recognized in two species, *R. homothallicus* and *R. microsporus*, the latter with three additional varieties : var. *chinenesis*, var. *oligosporus* and var. *rhizopodiformis*. And, judging from their general morphology and maximum growth temperature, *R. homothallicus* and *R. microsporus* are closely related. But, *R. homothallicus* and *R. microsporus* var. *oligosorus* were separated in cluster analysis in our results.

As a results of this study, *R. sexualis* ATCC 42542 and *R. oryzae* IFO 4772 would be revised. And the genus *Rhizopus* might be divided into four groups; *R. oryzae*, *R. microsporous*, *R. homothallicus* and *R. stolonifers* groups at the level of ITS sequence. Due to the lack of molecular taxonomic information about the genus *Rhizopus*, the information provided by these studies will be useful in evaluating the existing taxonomy of *Rhizopus*.

Acknoledgement

This work was funded by SangMyung University 2003.

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